Investigations on ruminal degradation of nutrients and feeding values of single feeds and compound feeds for cattle

Goran Grubješić

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Investigations on ruminal degradation of nutrients and feeding values of single feeds and compound feeds for cattle

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LIST OF ABBREVIATIONS

with the exception of abbreviations presented in Manuscripts 1–3 and for units defined by the International System of Units

ADFom	Acid detergent fibre expressed exclusive of residual ash
CA	Crude ash
CF	Crude fibre
CI	Confidence interval
CNCPS	Cornell Net Carbohydrate and Protein System
A	Fraction A: the non-protein N (**NPN**)
B1, B2, B3	Fraction B: the true protein differing in their rate of ruminal degradation
C	Fraction C: the acid detergent insoluble nitrogen (**ADIN**)
CP	Crude protein
DM	Dry matter
dOM	Digestible organic matter
ED_{CNCPS}	Effective ruminal degradation of CP calculated from CNCPS CP fractions
ED_{InsP6}	Effective ruminal degradation of $InsP_6$ determined *in situ*
ED_{IN_SITU}	Effective ruminal degradation determined *in situ*
a	Rapidly degradable fraction
b	Potentially degradable fraction
a*+*b	Maximal degradable fraction
c	Rate of degradation
k	Ruminal passage rate
EE	Ether extract
GP	Gas production
***b*GP**	Potential gas production
***c*GP**	Rate of gas production
GP_{24}	Corrected gas production at 24 h
ID_{RUP}	Intestinal digestibility of protein
$InsP_6$	Phytic acid (myo-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate)
ME	Metabolisable energy
N	Nitrogen
R^2	Coefficient of determination
RDP	Rumen degraded protein
RMSE	Root mean square error
RUP	Rumen undegraded protein
SEM	Standard error of the mean
ST	Starch
uCP	Utilisable crude protein at the duodenum

1. INTRODUCTION

The global human population is projected to increase to 9.7 billion in 2050 and 11.2 billion in 2100 (UN, 2019). This increase has to be met with intensification of food production to satisfy the growing needs for affordable and safe food. The global milk production was estimated at 843 million tonnes for the year 2018, which is a 2.2% increase from 2017 (FAO, 2019a). This growth was driven in the European Union (**EU**) by an increased milk yield per cow. Milk production in the EU alone was 167.3 million tonnes for the year 2018; a 1% increase from 2017.

However, intensification of global dairy production has critical impact on the environment due to the greenhouse gas production increase (FAO, 2019b). Further, incomplete digestion of crude protein (**CP**) leads to excretion of undigested nitrogen (**N**), resulting in big environmental pressure (FAO, 2019c). European Commission accused Germany in 2016 of violating EU wide nitrates directive (91/676/EEC), infringing upon ground water pollution caused by nitrates from agricultural sources. Dairy cows are known to have a relatively low efficiency of N utilisation (Dijkstra *et al*., 2013), thus contributing to the soil and water pollution.

One of the crucial strategies to lower the environmental impact of dairy cow production is accurate formulation of the feed ration, with a goal of better N utilisation. Another goal of correct feed formulation is the synchronous degradation of CP and the main feed energy source, starch (**ST**). This allows for optimal ruminal microbial growth and lowers the risk of sub-acute ruminal acidosis that commonly happens due to oversupply of easily fermentable energy (Enemark, 2008). This occurs commonly in high-yielding dairy cows that have high CP and energy requirements.

For accurate formulation of diets and satisfying the nutritive requirements of dairy cows, profound information on ruminal nutrient degradation and feeding value of single feeds is necessary. It is also essential to have the knowledge of any possible interactions among single feeds in feed mixtures that could affect the accuracy of diet formulation. The main goal of the present thesis was to evaluate possible interactions among single concentrate feeds that could affect ruminal degradation of nutrients and feeding values of compound feeds made thereof.

2. BACKGROUND

To satisfy the nutrient requirements of high-yielding dairy cows, single concentrates and compound feeds are commonly fed together with forages in the form of total mixed rations (**TMR**). While studies on feeding values of single feeds are common, information on possible interactions (**associative effects**) of feeding values of single feeds in compound feeds are scarce. Therefore, most feeding systems provide values for single feeds assuming they will be **additive** when mixed. However, this may not hold true for concentrate compound feeds.

Early research on the topic of additivity investigated if feeding values of animal feeds are fundamentally variable. The earliest known published research (Forbes *et al*., 1931) found that the net energy value of maize meal when fed together with alfalfa hay was higher than when fed alone. Forbes (1933) examined net energy value of different single concentrate feeds and forages using respiration calorimetry. The net energy of the whole ration differed from that of the sum of individual feed components, and therefore the former was recommended for the accurate estimation of the net energy supply to the animal. Most studies on additivity that followed focused on associative effects between forages and concentrates (Mould, 1982). Research on associative effects among single concentrate feeds is scarce. Blaxter *et al.* (1962) discussed the existence of associative effects, considering them too small to be accounted for in practice. In the following decades, major feeding systems both in Europe and the United States assumed additivity of feeds (Wood and Thorne, 2000; GfE, 2001). However, it is not well understood if additivity holds true for mixtures of single concentrate feeds.

Concentrate compound feeds are the main source of CP (CP = N · 6.25) and ST in dairy cow feeding when milk yield is high. Cows are ruminant herbivores, with complex multi-chamber stomachs having different and unique roles (Erickson *et al*., 2015). For characterisation of the protein value of feeds, contents of rumen degradable protein (**RDP**) and rumen undegradable protein (**RUP**) have to be determined. The fate of these two fractions is distinct. The RDP is degraded to peptides, amino acids (**AA**) and ammonia and utilised mostly by microbes residing in the rumen for creation of microbial CP (**MCP**) or absorbed through the rumen wall. The RUP leaves the rumen intact. The RUP fraction, together with the MCP is the basis for utilisable CP at the duodenum (**uCP**), as defined in the German protein evaluation system (GfE, 2001).

For determination of RUP, the *in situ* method is considered as a reference method (NRC, 2001; Südekum, 2005). Briefly, the *in situ* method uses ruminally fistulated animals for direct access to the rumen, with the goal of incubating small samples of feed in polyester bags over multiple time points. Equations (Ørskov and McDonald, 1979) are used to calculate the effective rumen degradation (**ED**) of a specific nutrient at a given passage rate (***k***). However, not only the amount of RUP supplied to the intestine is important, but also its digestibility and AA absorbability. The MCP and RUP differ in intestinal digestibility. While the nutritive quality of MCP is considered to be relatively constant and high, the intestinal digestibility of RUP (**ID_{RUP}**) differs among feeds (Stern *et al*., 1985). The ID_{RUP} can be estimated using a mobile bag technique or a three-step *in situ* and *in vitro* approach (Calsamiglia and Stern, 1995; NRC, 2001).

Production of MCP from available N sources requires an adequate and simultaneous supply of energy. To estimate energy values of feeds, *in vitro* methods based on gas production (**GP**) are commonly utilised (Menke and Steingass, 1988). This enables for fast measurement of GP, and subsequent calculation of digestibility of organic matter (**dOM**) and metabolisable energy (**ME**). These characteristics depend on intrinsic properties of ST and other carbohydrates, as well as CP and fat, and also on interactions between CP and ST in feeds (D'Mello, 2000).

Compound feeds are commonly provided in two physical forms: mash and pellet. Mash represents a mixture of ingredients in meal form, while pelleting represents a type of feed processing that mechanically agglomerates feed, using pressure (AAFCO, 2000). Steam can be applied as part of the conditioning before pelleting. Heat is commonly produced during pelleting due to physical movement of feed particles through dye openings. This can result in changes to the physical structure of protein and ST (Ljøkjel *et al*., 2003; Svihus *et al*., 2005; Svihus and Zimonja, 2011), affecting degradability and digestibility of single and compound feeds, to different extents.

Another important nutrient in dairy cow diets is phosphorus (**P**). It is an essential element playing a crucial role in both maintenance and productive requirements of dairy cows. Excretion of surplus P from dairy cows presents an increasing environmental concern (Knowlton *et al*., 2004). Further, due to the scarcity of mineral P sources in the nature (Desmidt *et al*., 2015), increasing utilisation of P is an increasingly important aim in animal nutrition. Alternatively, organic P sources bound in myo-inositol phosphate (**InsP**) can be easily hydrolysed using digestive enzyme phytase produced by rumen microorganisms (Yanke *et al*., 1998). The InsP with six attached phosphate groups is phytic acid (*myo*-inositol (1,2,3,4,5,6) hexakis (dihydrogen phosphate); **$InsP_6$**). The ruminal $InsP_6$ degradation varies widely among single feeds (Haese *et al*., 2016), but the number of single feeds with known extent of $InsP_6$

degradation in the literature is low. For accurate estimation of $InsP_6$ degradation in compound feeds, an extension of the database on $InsP_6$ degradation of single feeds is necessary, as well as the knowledge on possible interactions among single feeds when mixed. Heat treatment may lower the $InsP_6$ degradation of single feeds (Konishi *et al*., 1999). Because heat is produced during pelleting procedure, the effects of pelleting of compound feeds on $InsP_6$ degradation should be examined.

Previously mentioned *in situ* and *in vitro* methods for estimation of feeding values of feeds are all reliant on access to ruminally fistulated cows as donor of rumen fluid. There have been attempts to circumvent this by using only simple chemical analyses to estimate nutritive value of feeds, with implementation of specific mathematical models. One important model is the Cornell Net Carbohydrate and Protein System (**CNCPS,** Sniffen *et al*., 1992), relying on the chemical analysis described in Licitra *et al*. (1996) for protein fractionation. The CNCPS was used to predict *in situ* CP degradability (Shannak *et al*., 2000; Fox *et al*., 2003; Chrenková *et al*., 2014), and uCP (Zhao and Cao, 2004). If this method could be used to accurately predict protein values of a wide range of single and compound feeds, this would ease the workload and expense related to keeping ruminally fistulated cows and in particular regarding *in situ* incubations.

Based on the aforementioned, the aims of this doctoral work were:

- to evaluate the **additivity** of *in situ* determined values of EDCP, EDST, ED_{InsP6}, and *in vitro* determined values of GP, dOM, ME, uCP, ID_{RUP}, and CP fractions of single concentrate feeds in compound feeds, and:
- to evaluate the **effect of pelleting** on *in situ* determined values of EDCP, EDST, ED_{InsP6}, and *in vitro* determined values of GP, dOM, ME, uCP, ID_{RUP}, and CP fractions of compound feeds.

3. OVERVIEW OF INCLUDED MANUSCRIPTS

The **first aim** of the present thesis was to **evaluate additivity** of ruminal degradation of nutrients and feeding values of single concentrate feeds in compound feeds. The **second aim** was to **evaluate effects of pelleting** on ruminal degradation of nutrients and feeding value of compound feeds. Both aims were explored in all three manuscripts: using *in situ* ruminal degradation for values of $EDCP_{IN\_SITU}$, $EDST_{IN\_SITU}$ and related degradation parameters in Manuscript 1, and for ED_{InsP6} in Manuscript 3; and using different *in vitro* methods for values of GP, dOM, ME, uCP, ID_{RUP}, and CP fractions in Manuscript 2.

MANUSCRIPT 1: Determination of *in situ* ruminal crude protein and starch degradation values of compound feeds from single feeds

Published in *Archives of Animal Nutrition*

Ruminal degradation of CP and ST from different single and compound feeds were determined in dairy cows. Samples of 12 single feeds, 8 compound feeds in mash form, and 8 compound feeds in pelleted form were placed into polyester bags. Bags were incubated in the rumen of three ruminally fistulated lactating cows for 2, 4, 6, 8, 16, 24, 48, and 72 h. The degradation characteristics of single and compound feeds were computed. Observed degradation characteristics of single feeds were then used to calculate degradation characteristics of compound feeds, and such *calculated* values of compound feeds were statistically compared with *observed* values of compound feeds. Degradation characteristics of *mash* and *pelleted* compound feeds were also statistically compared. For deeper interpretation of *in situ* results, particle size determination was performed using the wet sieving technique.

- The first objective of this manuscript was to evaluate additivity of degradation characteristics of CP and ST from single feeds in compound feeds. Differences between calculated and observed EDCP and EDST values were considered to be small for practical feed formulation, and therefore the **additivity of ruminal degradation of CP and ST** of single feeds in compound feeds **was considered to be given**.
- The second objective was to evaluate effects of pelleting on degradation characteristics of CP and ST. **Pelleting had a small effect on EDCP and EDST**, presumably due to heat produced during processing not being excessively high. **Small increase in ED values** after pelleting was **attributed to an increase in**

share of fine particles as a result of pelleting that presumably left the *in situ* bags undegraded, rather than the change in CP or ST degradability.

MANUSCRIPT 2: Ruminal fermentation characteristics and related feeding values of compound feeds and their constituting single feeds studied by using *in vitro* techniques

Published in *Animal*

Ruminal fermentation characteristics and related feeding values of feed samples used in Manuscript 1 were analysed using different *in vitro* methods (Hohenheim gas test, extended Hohenheim gas test, three-step enzymatic digestibility method) and chemical protein fractionation (CNCPS).

- The first objective of this manuscript was to evaluate additivity of ruminal fermentation characteristics and dOM, ME, uCP, ID_{RUP}, and CP fractions. **Additivity was given for GP, dOM, ME, uCP, and CP fractions,** while **for ID_{RUP} the additivity was not given.**
- The second objective was to evaluate effects of pelleting on ruminal fermentation characteristics and dOM, ME, uCP, ID_{RUP}, and CP fractions. **Pelleting had only a small effect** on the ruminal fermentation characteristics and related feeding values of compound feeds.
- The third objective was to test the CNCPS model for prediction of $EDCP_{IN\_SITU}$. **Prediction of $EDCP_{IN\_SITU}$ values using the CNCPS model** for compound feeds used in the present thesis **was not accurate.**

MANUSCRIPT 3: Determination of *in situ* ruminal degradation of phytate from single and compound feeds using chemical analysis and NIRS

Published in *Animal*

Degradation of phytate from different single and compound feeds was evaluated in dairy cows. Samples of two chosen compound feeds (4 and 5) and all single feeds contained therein were incubated *in situ,* using the method described in Manuscript 1.

- The first objective of this manuscript was to characterise ruminal $InsP_6$ degradation of single and compound feeds, and evaluate additivity of ruminal $InsP_6$ degradation. The **additivity of ED_{InsP6}** values of single feeds was **given in mash compound feeds**, but **not in pelleted compound feeds**.
- The second objective was to use NIRS to predict $InsP_6$ concentrations in feed samples and *in situ* residues. **NIRS calibrations led to accurate estimation of $InsP_6$ concentrations**.

4. INCLUDED MANUSCRIPTS

4.1. Manuscript 1

Determination of *in situ* ruminal crude protein and starch degradation values of compound feeds from single feeds

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Determination of *in situ* ruminal crude protein and starch degradation values of compound feeds from single feeds

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Dairy cows are usually fed total mixed rations, consisting of forage, concentrates, and compound feed concentrates. Accurate formulation of compound feeds relies on the knowledge of possible associative effects between ruminal degradation characteristics of single feeds. Lacking that knowledge, additivity of ruminal degradation characteristics is often assumed. The main aim of this study was to evaluate the additivity of single feeds in compound feeds made thereof. To investigate this, 12 single feeds: maize, wheat, barley, soybeans, soybean meal, rapeseed meal, sunflower meal, faba beans, dried distillers' grains with solubles, maize gluten, wheat bran, and sugar beet pulp were used. Eight compound feeds with targeted crude protein (CP) concentrations (16, 18, 20, 22, 24, 26, 28, and 30% CP in dry matter) were produced, both in the mash and pelleted forms. Three ruminally fistulated dairy cows were used for *in situ* ruminal incubations. Effective ruminal degradation of CP and starch (ST) was computed for two ruminal feed passage rates of 0.05 h^{-1} (ED_5) and 0.08 h^{-1} (ED_8). The ED values of examined compound feeds could be, in most cases, accurately calculated from ED values of single feeds. Observed ED_5CP and ED_8CP were significantly lower than that calculated, but differences were small, up to 4 and 5 percentage points (pp), respectively. No significant difference was observed between calculated and observed ED_5ST and ED_8ST. The secondary goal of the study was to examine the effects of pelleting of compound feeds on *in situ* degradation characteristics. Pelleting significantly increased ED_5CP and ED_8CP (up to 6 and 8 pp, respectively), and ED_5ST and ED_8ST (up to 3 and 4 pp, respectively) of most compound feeds. This could have been caused by an increase in the proportion of fine feed particles because of pelleting, enabling them to leave the bags undegraded. It was concluded that small associative effects between the examined single feeds could be disregarded when formulating compound feeds for dairy cows, and that additivity of EDCP and EDST can be assumed in most cases.

Keywords: ruminants, protein degradation, starch degradation, additivity, associative effects, mixed feed

1. Introduction

Protein is an important but costly nutrient in farm animal nutrition. The efficiency of nitrogen (N) utilisation in ruminants is relatively low, underlying the importance of correct N balancing to minimise the loss of N and subsequent environmental pollution (Dijkstra et al. 2013). Thus, there are efforts to reduce crude protein (CP, 6.25 × N) concentration in dairy cow diets, while fulfilling requirements of high producing animals for both CP and energy. Starch (ST) is a major energy source in dairy cow diets and synchronisation of CP and ST rumen degradation is of great importance for microbial CP formation (Nocek and Russell 1988). It is, therefore, necessary to have extensive information about ruminal fermentation characteristics of CP and ST from different feed sources and their possible interactions in mixed feeds.

Diets of dairy cows consist of forage, single concentrates, and compound concentrates, provided as a total mixed ration or separately. Accurate formulation of compound feeds in accordance with animal feeding recommendations relies on the knowledge of ruminal degradation characteristics of single constituent feeds and on possible interactions between single feeds. Interactions between feeds are commonly termed associative effects. Existence of associative effects between concentrate feeds and forage in ruminants has long been studied (Forbes 1933, Mould 1982); however, research on additivity of single feeds in compound concentrates is less common.

For the estimation of rumen degradation characteristics, the *in situ* procedure is a widely used method (Südekum 2005). Feed samples are incubated in the rumen using polyester bags over multiple time points in ruminally fistulated dairy cows. The resulting effective degradability (ED) values provide insight into ruminally degraded and undegraded fractions of nutrients, such as CP and ST. Studies on additivity of CP and dry matter (DM) (Vik-Mo and Lindberg 1985; Murphy and Kennelly 1987; Chapoutot et al. 1990) have shown that accurate calculation of degradability of binary feed mixtures from single feeds is possible. Additivity of ruminal ST degradation has only been studied by Goelema et al. (1999) in pressure toasted single feeds and mixtures. In their experiment, observed effective degradability of starch (EDST) values were higher than those calculated, and differences increased with incubation time. Compound concentrates for cattle usually contain more than two single feeds. Whether or not degradation characteristics of single feeds are reflected in mixtures of more than two concentrates has not been investigated.

Compound feeds are used in mash form or as pellets. Pelleting includes exposure to moist heat, which can affect degradation characteristics of the compound feeds. Pelleting is known to improve handling properties (Svihus and Zimonja 2011), reduce the EDCP and increase the EDST (Ljøkjel et al. 2003) of some single concentrate feeds.

Effects of processing on starch nutritive value were reviewed in Svihus et al. (2005). Still, the understanding of the effect pelleting has on interactions between single feeds in compound feeds is insufficient.

Hence, the objectives of this study were to investigate:

(1) additivity of EDCP and EDST of single feeds in compound feeds, and

(2) effects of pelleting on EDCP and EDST of compound feeds.

It was hypothesised that the presence of associative effects would not be significant when single feeds are mixed together into compound feeds in mash form and that pelleting of compound feeds would lower EDCP and increase EDST.

2. Materials and Methods

2.1. Samples

Twelve common single feeds: maize, wheat, barley, soybeans, soybean meal, rapeseed meal, sunflower meal, faba beans, dried distillers' grains with solubles (DDGS), maize gluten, wheat bran, and sugar beet pulp were used for the formulation of eight compound cattle feeds targeting different CP concentrations. The intended CP concentrations were 16, 18, 20, 22, 24, 26, 28, and 30% CP in DM. Compound feeds were labelled from 1 to 8 based on increasing CP concentration (Table 1).

[Table 1. near here]

Production of compound feeds was completed under standard industrial conditions in a commercial feed mill [Raiffeisen Kraftfutterwerk (RKW) Kehl, Germany]. Single feeds were ground through a 3 mm sieve (mill: Tietjen VD10; Hemdingen, Germany) and mixed into the eight compound feeds into 3000 kg batches (mixer: Bühler Speed Mix 6300; Uzwil, Switzerland). A part of each batch was processed by conditioning with steam for 10–15 s and pelleted at a temperature of 50–60°C (5 mm matrix die size and 70 mm thickness; pellet mill: 7726, CPM Crawfordsville, USA,) with an exit temperature of 80–90°C.

The chemical composition of single feeds varied considerably (Table 2). Crude protein concentration varied from 87 g/kg DM in maize to 479 g/kg DM in soybean meal. Starch concentration varied from 158 g/kg DM in wheat bran to 644 g/kg DM in maize. All other nutrient concentrations were in the expected range for single feeds. Targeted CP concentrations were achieved in all compound feeds. Overall, analysed nutrient concentrations in mash and pelleted feed were very similar, with the exception of CP and ST concentration in compound feed 6.

[Table 2. near here]

2.2. In situ *procedure*

The study was conducted in accordance with the German animal welfare regulations and approved by the Animal Welfare Authority (Regierungspräsidium Stuttgart, Germany). Three lactating dairy cows (Jerseys) fitted with a rumen cannula, with an average milk yield of 18 l/day and an average body weight of 532 kg were used. Cows were provided a total mixed ration consisting of 25% concentrate mixture (27% barley, maize, and faba beans each, and 20% peas), 24% maize silage, 24% grass silage, 17% meadow hay, 5% rapeseed meal, 2% barley straw, and 2% mineral additives for *ad libitum* consumption. The average DM intake was 16.4 kg per day.

In situ incubations were conducted as described by Seifried et al. (2016a). All single and compound feeds (28 in total) were treated the same way. Briefly, samples were ground to pass through a 2 mm sieve (mill: SM 1, Retsch GmbH, Haan, Germany) and 8 g (± 0.015 g) of each sample was weighed and placed into polyester bags (bag size of 10 × 20 cm, pore size of 50 µm, ANKOM Technology, USA). There were three to five incubation replicates per sample, incubation time, and animal, based on the expected amount of residue in the bags. Prior to incubation, bags were soaked in warm water (≈39°C) for up to 1 min. Bags were ruminally incubated for 2, 4, 6, 8, 16, 24, 48, and 72 h, and were subsequently subdued in ice-cold water. Bags were then washed manually with tap water and frozen (-20°C). Upon thawing, bags were washed in a domestic washing machine (model: W classic, Miele & Cie. KG, Gütersloh, Germany) using cold water without detergent for a duration of 17 min without centrifugation. Zero-hour bags were only washed, without incubation, in three replicates per feed. After being washed, bags were dried overnight at 60°C in an air-forced oven. Dried residues were weighed, pooled among the three to five replicate bags, and pulverised (mill: Pulverisette, Fritsch GmbH, Idar-Oberstein, Germany) in preparation for chemical analyses of DM, CP, and ST.

2.3. Chemical analyses

Single and compound feed samples were analysed for crude nutrients according to the official methods in Germany [Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten (VDLUFA) 2007], determining DM (method 3.1), CP (method 4.1.1), ether extract (EE) (method 5.1.1), neutral detergent fibre assayed with a heat stable amylase exclusive of residual ash (aNDFom) (method 6.5.1), acid detergent fibre

exclusive of residual ash (ADFom) (method 6.5.2), and crude ash (CA) (method 8.1). Starch concentrations in samples and bag residues were analysed enzymatically as described by Seifried et al. (2016b).

Near-infrared spectroscopy (NIRS) was used for determination of CP concentration in all feeds and residues obtained from the bags. Starch concentration was determined in ST-rich feeds (maize, wheat, barley, faba beans, maize gluten, and wheat bran) and all compound feeds, and the residues from corresponding bags. In addition, one third (one randomised animal per feed sample and incubation time) of all bag residues (268 samples for N and 184 samples for ST) were chemically analysed for CP and ST using the aforementioned methods. These samples were used to expand the NIRS database of Krieg et al. (2018a), leading to a database of 888 samples for N (674 for calibration, 214 for validation) and 794 samples for ST (588 for calibration, 206 for validation). Reference values of the extended database ranged between 0.2 and 11.2% DM for N and 0.5 to 76.5% DM for ST. Spectra recording (SpectraStar 2500X, Software: Unity InfoStar Version 3.11.1; Unity Scientific, Brookfield, CT, USA), spectral pre-treatment, and calibration development (Ucalibrate Version: 3.0.0.23; Unity Scientific) were conducted as described by Krieg et al. (2018a). The best performance was achieved using the wavelength segment of 680-2500 nm and the 2^{nd} (N) and the 1^{st} (ST) derivative of the spectra. The used calibrations had a standard error of prediction of 0.16% DM (N) and 2.20% DM (ST). The coefficient of variation and the slope of the validation step were > 0.99 for both constituents. More detailed information about the established calibrations is provided in Annex 1.

2.4. Determination of particle size distributions

Seven grams of feed, ground to pass through a 2 mm sieve, was dissolved in distilled water at room temperature for 60 min in three replicates. A sieve shaker (AS200, Retsch GmbH, Germany) with 7 sieves containing sieve sizes of 2.000, 1.180, 1.000, 0.500, 0.250, 0.125, and 0.063 mm was used for the wet sieving procedure. Because of clogging of the smallest (0.063 mm) sieve when sieving pelleted compound feeds, for those samples only sieves with the size of 2.000, 1.180, 1.000, 0.500, 0.250, and 0.125 mm were used. Each sieving run lasted 12 min alternating between 10 s of shaking and 2 s pause, utilising 2.5 L of water per min. Contents of each sieve were transferred into filters (MN 615 ø240 mm, Macherey-Nagel GmbH & Co. KG, Germany), dried overnight, and weighed.

2.5. Calculations

2.5.1. In situ

A model including lag time (Ørskov and McDonald 1979) was used to describe the ruminal degradation kinetics of CP and ST (eq. 1) and calculated using GraphPad Prism software (version 5.0, GraphPad Software Inc., CA, USA). Therein *a* (%) represents the rapidly degradable fraction, *b* (%) is the potentially degradable fraction, *c* (% h^{-1}) is the rate of degradation, *t* (h) is time after the start of incubation, and *lag* (h) is the lag time.

$$Y = a + b \cdot [1 - e^{-c \cdot (t \cdot lag)}] \quad (1)$$

The following equation of McDonald (1981) modified by Südekum (2005) was used to compute the EDCP and EDST (%) assuming feed passage rates in the rumen (*k*) of 0.05 h^{-1} (ED_5) and 0.08 h^{-1} (ED_8).

$$ED = a + \left(\frac{b \cdot c}{c + k}\right) \cdot (e^{-k \cdot lag}) \quad (2)$$

Degradation characteristics computed this way were termed 'observed'. In addition, ruminal degradation characteristics of compound feeds were calculated from observed values of single feeds. For this purpose, degradation characteristics of single feeds were weighted based on their CP or ST contribution to the total CP or ST of the respective compound feed and termed 'calculated'.

$$dCF_x calc = [\,(dSF_1 obs \cdot w_1) + (dSF_2 obs \cdot w_2) + \ldots + (dSF_i obs \cdot w_i)\,] / 100 \quad (3)$$

$dCF_x calc$ = calculated degradation characteristics (*a, b, c*, ED_5, or ED_8) of compound feed *x*
$dSF_i obs$ = observed degradation characteristic (*a, b, c*, ED_5, or ED_8) of single feed *i*
w_i = weighted CP or ST contribution of single feed *i* to total CP or ST pool of compound feed *x*

This calculation was completed separately for each of the three cows, and the cow was used as the experimental unit in statistical analyses. Significant differences between calculated and observed values of degradation characteristics were considered as associative effects and expressed in percentage points (pp).

2.6. Statistical analyses

Calculated and observed degradation characteristics of compound feeds, as well as degradation characteristics of mash and pelleted compound feeds were compared using the procedure MIXED (version 9.4 of SAS system for Windows, SAS Institute, NC, USA). The model contained the compound feed (1–8) and the way values were obtained (calculated, observed) or pelleting (mash, pellet), and their interaction with compound feed as fixed effects, whereas the animal was considered a random effect.

3. Results

3.1. Ruminal degradation characteristics of single feeds

Degradation characteristics of single feeds are presented in Table 3. Both CP and ST were potentially almost completely degradable (*a*+*b*CP and *a*+*b*ST ranged from 94% to 100%). The values of ED_5CP and ED_8CP were lowest in maize (70% and 61%, respectively), and highest in faba beans (92% and 89%, respectively). The ED_5ST and ED_8ST varied from 78% and 70%, respectively, in maize and 98% in wheat bran. The lag time of CP and ST degradation, as well as estimates of *a*, *b*, and *c*, varied widely among single feeds.

[Table 3. near here]

3.2. Calculated and observed ruminal degradation characteristics of compound feeds

Calculated ED_5CP values of compound feeds ranged between 76% and 87% and ED_8CP between 69% and 83% (Table 4). Statistically significant interactions between the way values are obtained (calculated vs. observed) and the compound feeds were found for both ED_5CP and ED_8CP. Observed ED_5CP and ED_8CP values were significantly lower than those calculated in compound feed 2, 4, and 5. However, differences did not exceed the value of 5 pp, which was the difference in compound feed 4 for k = 0.08 h^{-1}. Observed lag for CP degradation was significantly lower than that calculated. Calculated *c*CP values of compound feeds ranged between 17.2 h^{-1} in compound feed 5 and 26.5 h^{-1} in compound feed 3. Observed *c*CP was significantly lower than that calculated in compound feeds 1 (9.6 pp h^{-1}), 4 (9.4 pp h^{-1}), 5 (6.0 pp $^{h-1}$), 7 (4.5 pp h^{-1}), and 8 (4.9 pp h^{-1}). Observed *b*CP values were significantly

smaller than calculated in compound feed 2 and significantly higher than that calculated for compound feeds 3, 4, 5, 6, 7, and 8.

[Table 4. near here]

Calculated ED_5ST values of compound feeds ranged between 84% and 97% and ED_8ST values between 78% and 96%. The way values were obtained had no significant effect on EDST and differences between calculated and observed EDST were numerically small with a maximum of 2 pp. Calculated *c*ST values of compound feeds ranged between 30.7 h^{-1} in compound feed 5 and 236 h^{-1} in compound feed 7, whereas observed *c*ST values of compound feeds ranged between 13.1 h^{-1} in compound feed 5 and 3882 h^{-1} in compound feed 7. Because of some extreme values, the additivity of *c*ST was not statistically analysed. Even though the *c*ST value in compound feed 7 was an outlier (3882 h^{-1}), its effect on ED calculation was not detrimental for the purpose of additivity confirmation. Observed *b*ST was statistically smaller than that calculated in compound feeds 2, 3, 4, 5, 6, and 7.

3.3. Effect of pelleting on ruminal degradation characteristics of compound feeds

A significant interaction between pelleting and compound feed was noted for EDCP (Table 5). Pelleting significantly increased ED_5CP values in compound feeds 4 (79 vs. 85%), 5 (74 vs 79%), and 6 (80 vs. 83%). Pelleting significantly increased ED_8CP values in compound feeds 4 (72 vs. 80%), 5 (67 vs. 72%), and 6 (73 vs. 77%). The *c*CP and the lag of CP degradation were not affected by pelleting. Pelleting significantly affected *b*CP, with significant increases in compound feeds 1 and 3, but significant decreases in compound feeds 2, 4, 5, 6, 7, and 8.

Pelleting significantly affected ED_5ST (up to 3 pp) and ED_8ST (up to 4 pp) values in compound feeds. The *c*ST was not statistically analysed because of some extreme values. Pelleting significantly increased *b*ST, and numerically increased *a*ST values in all compound feeds.

[Table 5. near here]

3.4. Particle size distributions

Particle size distribution is presented in Table 6. Pelleting numerically increased the share of particles passing through smaller sieves (0.500, 0.250, and 0.125 mm) in all compound feeds.

[Table 6. near here]

4. Discussion

4.1. Additivity of ruminal degradation characteristics of single feeds

Overall, additivity of ruminal CP and ST degradation characteristics for single feeds was confirmed in the present study. A few statistical differences between calculated and observed degradation characteristics occurred, but only in one case did the difference in ED_8CP exceeded 3 pp. In most cases, differences were not significant. Vik-Mo and Lindberg (1985) and Murphy and Kennelly (1987) confirmed additivity of EDCP using binary concentrate mixtures. Even though compound feeds in the present study contained between five and seven single feeds, the associative effects were not higher than those found in the literature for binary concentrate mixtures. However, differences between calculated and observed values were overall higher for ED_8CP than for ED_5CP in the present study. Although the observed lag of CP degradation was significantly shorter than that the calculated lag, these differences did not lead to large numerical differences in EDCP. This is related to observed *c*CP values being significantly lower than those calculated in five of the compound feeds (1, 4, 5, 7, and 8), counteracting the lower lag time.

It was not possible to relate the detected differences between calculated and observed EDCP values to a specific single feed. Not all of the single feeds were present in all compound feeds. Ruminal degradation characteristics of single feeds were compared against previous studies and former values obtained at our institute for EDCP, namely, Prestløkken (1999), NRC (2001), Woods et al. (2003), Lund et al. (2008), Westreicher-Kristen et al. (2012), Habib et al. (2013), and Krieg et al. (2018b). Values for EDST were compared against Seifried et al. (2015) and Krieg et al. (2017). Both EDCP and EDST were compared with Goelema et al. (1999), Ljøkjel et al. (2003), Seifried et al. (2016a, 2016b), and Razzaghi et al. (2016). Values from listed studies were broadly in agreement with values found in the present study when accounting for methodological differences.

Hypothesised reasons for perceived associative effects in some compound feeds included physical conditions inside bags. Although single feeds were all ground through the same screen, they differed in particle size distribution (Table 6). Physical characteristics of single feeds may interact when mixed. The interaction of maize gluten in meal form with other feeds inside the bags because of its high viscosity, sticking when soaked, lowering the surface area for potential degradation is an example of such (Stern et al. 1983). On the other hand, increased viscosity could theoretically induce clogging of bag pores lowering ED, but no bloating in bags containing maize gluten alone or in compound feed was noted. Murphy and Kennelly (1987) indicated small differences between calculated and observed values of ED8CP in binary feed mixtures, up to 2 pp in barley-canola meal mixtures and up to 6 pp in barley-maize gluten meal mixtures. The possible influence of maize gluten on *in situ* degradation measurements should be further investigated.

No differences were found between calculated and observed values for ED_5ST and ED_8ST. The absence of any effect was likewise related to the almost complete ST degradation potential ($a+bST \geq 98\%$) and overall high EDST values (78-99% for k = 0.05 h^{-1} and 71-99% for k = 0.08 h^{-1}). Differences in the lag of ST degradation were different only between compound feeds but not between calculated and observed value data. We are not aware of similar studies exploring additivity of ST in concentrate feed mixtures.

Because the *c*ST value of wheat bran in the present study was unusually high, comparison with Cerneau and Michalet-Doreau (1991) is shown. Their parameters of *in situ* ST degradation of wheat bran were *a*ST = 83%, *c*ST = 25.4 % h^{-1}, and ED_6ST = 96%, whereas values in the present study were *a*ST = 80%, *c*ST = 1748 % h^{-1} and ED_5ST = 99%. Although the computed *c*ST value in our study was very high, it could be used to calculate ED values, and the resulting difference between calculated and observed EDST values was not significant. This indicates that additivity calculation should be completed for ED values instead of individual degradation parameters. However, the high *a*-fraction indicates that most of the starch is immediately washed out of the bag when incubated in the rumen and it is therefore questionable if the *in situ* procedure is an adequate method for measuring starch degradation of wheat bran (Seifried et al. 2015).

4.2. Effect of pelleting on ruminal degradation characteristics of compound feeds

Pelleting significantly increased EDCP in three out of eight compound feeds. Direct comparisons of pelleting effects on ruminal CP degradation of compound feeds with literature were not possible. However, the present results contrasted results from different processing and heat treatment effects on EDCP of single feeds (Goelema et al. 1999, Ljøkjel et al. 2003) and binary feed mixtures (Razzaghi 2016). Usually, the underlying reason for reduced EDCP because of pelleting is the effect of high temperature during processing, facilitating the destruction of the three-dimensional protein structure (Svihus and Zimonja 2011). In the present study, the pellet exit temperature of 80-90°C might not have been high enough to trigger such a response in examined compound feeds, such that other changes occurring during pelleting probably had a greater effect on EDCP, such as changes in particle size distribution of feed particles could increase the *a*-fraction, and thus, EDCP, as described later. Because pelleting did not significantly affect any other degradation characteristic, it is difficult to identify the general cause for significant EDCP differences between the three mash and pelleted compound feeds.

The correction for small particle loss (Weisbjerg et al. 1990) was completed but could not be used because the water-soluble N appeared higher than that of the washout fraction in some samples (soybean meal, rapeseed meal, sunflower meal, faba beans, and sugar beet pulp; mash compound feeds 6, 7, and 8). Soybeans and especially faba beans were dramatically slower to filter when compared to other samples. A correction for small particle loss could not be applied, and the question of whether pelleted compound feeds showed higher water-solubility (and thus higher ED) because of greater physical particle arose. Goelema et al. (1999) suggested that a decrease of average particle size because of pelleting affects degradability of CP in bags and that such decrease could be due to the washout of undegraded particles through the bag pores. The same reduction in particle size may affect ED of compound feeds to a variable extent, because of different degradation characteristics of single constituent feeds. It is known that increasing the fineness of grinding affects N degradability of concentrates, and that feeds ground through the same screen size differ in particle size (Michalet-Doreau and Cerneau 1991). Investigation of the particle size distribution of feed samples could be an invaluable tool in the detailed evaluation of loss from polyester bags (D'Mello 2000). Determination of particle size distribution was completed in the present study using a wet sieving procedure, which was preferred to dry sieving because it mimicked conditions in the rumen during the *in situ* procedure more closely (Kennedy 1984). Particle size determination in the present study indicated an increase in fine feed particles that could pass through smaller sieves, similar to

Abdollahi et al. (2011) who noted that pelleting increased the proportion of fine particles smaller than 0.075 mm. Because the pores in the polyester bags used in the present study had a diagonal of approximately 0.071 mm, it can be assumed that some fine particles contained in pellets could have been released upon milling in the preparation for *in situ* incubations and could pass through bag pores undegraded. Yet, because of the removal of the sieve with 0.063 mm pore size for pelleted compound feeds (because of clogging), the precise estimation of washout of particles smaller than the *in situ* bag pore size in pelleted compound feeds was impossible, rendering the extent of such theoretical passage in the present study unclear. As mentioned in the previous section, the microbial mass in bags could be a factor influencing results from *in situ* studies. However, there could also be a possible connection between the decrease in particle size (because of pelleting) and increased microbial colonization during bag incubation (D'Mello 2000), which could result in increased microbial activity in bags while in rumen fluid, and increased degradation of pelleted compound feeds.

Pelleting increased EDST up to 3 pp and 4 pp for k = 0.05 h^{-1} and k = 0.08 h^{-1}, respectively. Razzaghi et al. (2016) explored the effects of pelleting on binary mixtures of maize, wheat, soybean meal, and sugar beet pulp *in situ*. They found that overall, pelleting increased EDST in maize and maize mixtures, whereas it decreased EDST in wheat and wheat mixtures. In the present study, EDST of all compound feeds increased after pelleting. Heat treatment can lead to gelatinisation of starch and changes in the physical structure of the feed (Goelema et al. 1999), increasing the ST degradability. However, the relatively low moisture level of compound feeds in the present study (up to 11%) during pelleting, as well as the pellet exiting temperature (80-90°C) could limit the extent of starch gelatinisation (Ljøkjel et al. 2003), lowering the effect pelleting had on rumen starch degradation. The EDST was relatively high even before pelleting (average over compound feeds of 87%), and large numerical differences were less likely. Pelleting numerically increased the *a*ST in all compound feeds, up to 12 pp, indicating a similar mechanism of small particle release after pellets were ground, such as the case of CP degradation characteristics, and as indicated by decreasing average particle size as a result of pelleting.

5. Conclusions

Effective ruminal degradation of crude protein (EDCP) and starch (EDST) for compound feeds in mash form could be calculated from EDCP and EDST of constituent single feeds in most cases. Pelleting resulted in increased EDCP in some compound feeds, possibly because of more fine feed particles that could leave the bags undegraded. Pelleting increased EDST only to a low extent. Differences found were considered to be of low relevance for compound feed production.

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Disclosure statement

No potential conflict of interest is reported by the authors.

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Tables

Table 1. Single feed composition of the eight compound feeds [g/kg DM].

Single feed	Compound feed							
	1	2	3	4	5	6	7	8
Maize	454	217	-	96	316	95	-	-
Wheat	299	257	-	-	117	-	251	-
Barley	-	-	376	465	-	124	-	278
Soybeans	-	-	53	178	-	207	191	207
Soybean meal	97	-	-	49	79	-	63	155
Rapeseed meal	-	140	-	-	173	116	148	-
Sunflower meal	-	-	50	-	100	146	150	147
Faba beans	-	-	179	161	163	212	-	213
DDGS	-	84	-	51	52	-	-	-
Maize gluten	101	100	197	-	-	-	99	-
Wheat bran	50	99	146	-	-	-	98	-
Sugar beet pulp	-	103	-	-	-	99	-	-

Table 2. Analysed composition of single and compound feeds [g/kg DM, unless otherwise stated].

Feed		DM [g/kg]	CP*	ST*	CA	EE	aNDFom	ADFom
Maize		897	87	644	14	43	84	30
Wheat		887	140	544	19	31	86	36
Barley		894	130	467	28	33	162	76
Soybeans		948	391	-†	56	221	109	65
Soybean meal		901	479	-	74	26	111	66
Rapeseed meal		898	370	-	92	38	304	223
Sunflower meal		906	321	-	78	30	402	307
Faba beans		891	267	294	40	23	173	129
DDGS		919	317	-	64	81	338	198
Maize gluten		888	169	159	84	36	343	95
Wheat bran		906	186	158	59	55	398	135
Sugar beet pulp		945	94	-	85	19	347	174
1	mash	893	158	486	31	40	141	45
	pellet	901	158	463	33	43	142	50
2	mash	901	174	293	49	41	232	105
	pellet	896	174	295	50	41	233	106
3	mash	895	188	294	50	44	244	110
	pellet	893	187	297	50	43	264	117
4	mash	901	213	358	36	72	145	76
	pellet	896	204	357	38	60	160	74
5	mash	892	229	329	45	43	198	127
	pellet	895	228	321	46	47	189	111
6	mash	908	253	198	53	72	232	146
	pellet	897	229	259	50	54	220	131
7	mash	900	277	187	59	74	250	135
	pellet	900	261	202	59	65	237	133
8	mash	901	291	217	50	69	185	119
	pellet	897	291	231	50	63	183	115

DM = dry matter; CP = crude protein; ST = starch; CA = crude ash; EE = ether extract; aNDFom = neutral detergent fibre assayed with a heat stable amylase, exclusive of residual ash; ADFom = acid detergent fibre, exclusive of residual ash; * Determined using near-infrared spectroscopy; † Not determined.

Table 3. Ruminal crude protein (CP) and starch (ST) degradation characteristics of single feeds (n = 3 cows).

Feed	*a*	*b*		*c*		*lag*		ED_5		ED_8	
	mean	mean	*SD*	mean	*SD*	mean	*SD*	mean	*SD*	mean	*SD*
CP											
Maize	27	73	0.0	8.2	2.24	1.0	1.02	70	5.7	61	6.2
Wheat	52	45	0.8	35.4	6.43	2.8	0.86	86	1.8	81	2.4
Barley	34	60	0.9	33.0	6.71	0.0	0.00	86	0.9	82	1.4
Soybeans	50	50	0.0	13.7	5.14	2.7	2.41	81	0.9	75	1.6
Soybean meal	12	88	0.1	13.2	3.68	3.0	0.89	66	2.6	55	2.7
Rapeseed meal	22	73	0.5	11.6	0.87	0.6	0.51	72	0.6	63	0.7
Sunflower meal	20	76	0.6	19.3	4.11	0.2	0.24	79	1.3	72	1.7
Faba beans	61	37	0.2	28.2	6.95	0.4	0.32	92	0.8	89	1.0
DDGS	53	41	1.5	12.2	2.94	0.0	0.00	82	0.8	78	1.3
Maize gluten	60	35	0.3	26.4	3.27	0.0	0.00	89	0.5	87	0.7
Wheat bran	45	49	0.4	26.3	1.15	0.2	0.08	85	0.6	82	0.7
Sugar beet pulp	19	77	0.3	11.4	1.70	0.0	0.01	72	2.3	64	2.7
ST											
Maize	33	67	0.0	10.4	2.38	0.2	0.26	78	4.0	70	4.5
Wheat	75	25	0.1	90.2	12.98	0.0	0.00	98	0.1	97	0.2
Barley	35	63	0.4	79.2	9.82	0.0	0.00	94	0.5	92	0.7
Soybeans	*	-	-	-	-	-	-	-	-	-	-
Soybean meal	-	-	-	-	-	-	-	-	-	-	-
Rapeseed meal	-	-	-	-	-	-	-	-	-	-	-
Sunflower meal	-	-	-	-	-	-	-	-	-	-	-
Faba beans	59	41	0.3	38.3	6.64	1.3	0.28	93	1.3	89	1.7
DDGS	-	-	-	-	-	-	-	-	-	-	-
Maize gluten	53	45	0.3	21.6	4.35	0.0	0.00	90	1.6	86	2.0
Wheat bran	80	19	0.6	1748	2259	1.0	0.86	98	0.4	98	0.9
Sugar beet pulp	-	-	-	-	-	-	-	-	-	-	-

a = rapidly degradable fraction [%]; *b* = potentially degradable fraction [%]; *c* = rate of degradation of *b* [h^{-1}]; *lag* = lag time [h]; ED = effective degradation [%] of CP or ST at a passage rate of 0.05 h^{-1} (ED_5) and 0.08 h^{-1} (ED_8); * Not determined.

Table 4. Comparison of calculated and observed ruminal crude protein (CP) and starch (ST) degradation characteristics of compound feeds in mash form (n = 3 cows).

		CP						ST					
		a	*b*	*c*	*lag*	ED_5	ED_8	*a*	*b*	*c*	*lag*	ED_5	ED_8
1	calculated	34	64^{cd}	20.2^{bc}	1.9	76^{gh}	69^{gh}	49	51^{g}	66.4	0.1	85	80
	observed	35	64^{c}	10.6^{h}	0.8	77^{fg}	69^{fg}	48	52^{fg}	14.8	0.1	86	81
2	calculated	39	57^{g}	19.1^{bcd}	0.8	79^{de}	73^{cd}	55	44^{j}	133.8	0.1	88	85
	observed	40	54^{h}	16.1^{defg}	0.0	81^{bc}	76^{b}	63	37^{k}	13.2	0.1	90	86
3	calculated	47	49^{j}	26.5^{a}	0.4	87^{a}	83^{a}	45	54^{e}	201.0	0.3	94	91
	observed	45	51^{i}	26.7^{a}	0.1	88^{a}	84^{a}	53	46^{i}	53.7	0.0	95	92
4	calculated	43	54^{h}	21.4^{b}	1.3	83^{b}	77^{b}	38	61^{a}	60.2	0.2	91	88
	observed	37	61^{ef}	12.0^{gh}	0.1	79^{de}	72^{d}	39	60^{b}	41.2	0.0	91	88
5	calculated	32	65^{c}	17.2^{cdef}	1.0	77^{efg}	70^{efg}	46	54^{e}	30.7	0.3	84	78
	observed	30	67^{ab}	11.2^{h}	0.5	74^{h}	67^{h}	48	52^{f}	13.1	0.8	84	78
6	calculated	40	58^{g}	18.4^{bcde}	1.1	81^{bc}	75^{bc}	43	57^{c}	41.9	0.5	88	84
	observed	37	60^{f}	14.5^{efgh}	0.6	80^{cd}	73^{cd}	49	49^{h}	30.9	1.1	88	84
7	calculated	36	61^{fe}	18.5^{bcde}	1.6	79^{def}	72^{ed}	73	26^{l}	236.0	0.1	97	96
	observed	32	65^{bc}	14.0^{gfh}	0.4	78^{defg}	71^{def}	82	17^{m}	3882	0.0	99	99
8	calculated	36	62^{de}	19.6^{bcd}	1.6	80^{cd}	73^{cd}	43	56^{cd}	65.9	0.4	94	91
	observed	30	68^{a}	14.7^{efgh}	0.7	79^{de}	72^{de}	43	56^{d}	239.7	0.8	95	92
Pooled SEM		-	0.7	1.64	-	0.9	1.0	-	0.3	-	-	-	-
CF	1	35			1.3^{a}			49		40.6	0.1^{bc}	86^{e}	81^{e}
	2	40			0.4^{cd}			59		73.5	0.1^{bc}	89^{d}	85^{d}
	3	46			0.3^{d}			49		127.3	0.2^{bc}	94^{b}	92^{b}
	4	40			0.7^{bcd}			38		50.7	0.1^{bc}	91^{c}	88^{c}
	5	31			0.8^{bc}			47		21.9	0.6^{ab}	84^{f}	78^{f}
	6	38			0.8^{bc}			46		36.4	0.8^{a}	88^{d}	84^{d}
	7	34			1.0^{ab}			77		2059	0.1^{c}	98^{a}	97^{a}
	8	33			1.1^{ab}			43		152.8	0.6^{ab}	94^{b}	92^{b}
Pooled SEM		-			0.17			-		-	0.18	0.9	1.1
W	calculated	38			1.2^{a}			49		104.5	0.28	90	87
	observed	36			0.4^{b}			53		536.1	0.37	91	87
Pooled SEM		-			0.10			-		-	-	-	-
p-values	CF × W	-	<0.001	0.035	0.463	0.009	0.002	-	<0.001	-	0.529	0.816	0.679
	CF	-	<0.001	<0.001	0.001	<0.001	<0.001	-	<0.001	-	0.026	<0.001	<0.001
	W	-	<0.001	<0.001	<0.001	0.101	0.062	-	<0.001	-	0.464	0.061	0.139

a = rapidly degradable fraction [%]; *b* = potentially degradable fraction [%]; *c* = rate of degradation of *b* [h^{-1}]; *lag* = lag time [h]; ED = effective degradation [%] of CP or ST at a passage rate of 0.05 h^{-1} (ED_5) and 0.08 h^{-1} (ED_8). Different superscripts within a column and main effect (or their interaction) indicate significant differences. *p*-values for main effects and their interactions are shown: the way values were obtained (W: calculated and observed) and compound feeds (CF: 1-8). Observed values in Table 4 are identical to mash values in Table 5, named respective to comparisons being made.

Table 5. Effects of pelleting on crude protein (CP) and starch (ST) degradation characteristics of compound feeds (n = 3 cows).

			CP						ST					
			a	*b*	*c*	*lag*	ED_5	ED_8	*a*	*b*	*c*	*lag*	ED_5	ED_8
1	mash	35	64^{c}	10.6	0.8	77^{gh}	69^{jk}	48	52^{c}	14.8	0.1	86	81
	pellet	31	66^{ab}	12.7	0.1	77^{gh}	70^{ij}	53	47^{f}	17.0	0.0	88	84
2	mash	40	54^{gh}	16.1	0.0	81^{de}	76^{def}	63	37^{i}	13.2	0.1	90	86
	pellet	44	51^{i}	17.7	0.1	83^{dc}	78^{cd}	69	30^{j}	14.7	0.0	91	88
3	mash	45	51^{i}	26.7	0.1	88^{a}	84^{a}	53	46^{f}	53.7	0.0	95	92
	pellet	40	56^{fg}	28.9	0.2	86^{ab}	82^{ab}	56	42^{h}	54.7	0.1	95	93
4	mash	37	61^{d}	12.0	0.1	79^{efg}	72^{ghi}	39	60^{a}	41.2	0.0	91	88
	pellet	45	52^{hi}	16.1	0.0	85^{bc}	80^{bc}	48	50^{d}	45.1	0.0	93	90
5	mash	30	67^{ab}	11.2	0.5	74^{h}	67^{k}	48	52^{c}	13.1	0.8	84	78
	pellet	40	58^{e}	11.1	0.4	79^{efg}	72^{ghi}	57	43^{h}	11.8	0.4	86	82
6	mash	37	60^{d}	14.5	0.6	80^{ef}	73^{fgh}	49	49^{e}	30.9	1.1	88	84
	pellet	45	52^{hi}	13.4	0.1	83^{cd}	77^{cde}	62	38^{i}	16.8	0.0	91	87
7	mash	32	65^{bc}	14.0	0.4	78^{fg}	71^{hij}	82	17^{k}	3882	0.0	99	99
	pellet	42	56^{ef}	12.1	0.6	80^{def}	74^{fgh}	83	16^{l}	1717	0.7	98	97
8	mash	30	68^{a}	14.7	0.7	79^{efg}	72^{ghij}	43	56^{b}	239.7	0.8	95	92
	pellet	43	57^{ef}	12.0	0.9	81^{de}	74^{efg}	55	44^{g}	244.7	0.8	96	94
Pooled SEM		-	0.8	-	-	1.0	1.2	-	0.4	-	-	-	-
CF	1	33			11.7^{c}	0.5^{ab}			51		15.9	0.1	87^{e}	83^{e}
	2	42			16.9^{b}	0.1^{c}			66		13.9	0.1	90^{d}	87^{d}
	3	42			27.8^{a}	0.2^{bc}			55		54.2	0.0	95^{b}	93^{b}
	4	41			14.1^{bc}	0.1^{c}			43		43.1	0.0	92^{c}	89^{c}
	5	35			11.2^{c}	0.5^{ab}			52		12.4	0.6	85^{f}	80^{f}
	6	41			14.0^{bc}	0.3^{bc}			56		23.9	0.6	89^{d}	85^{d}
	7	37			13.1^{bc}	0.5^{ab}			82		2800	0.4	98^{a}	98^{a}
	8	36			13.3^{bc}	0.8^{a}			49		242.2	0.8	95^{b}	93^{b}
Pooled SEM		-			1.94	0.14			-		-	-	0.8	1.1
P	mash	36			15.0	0.4			53		536.1	0.37	91^{a}	87^{a}
	pellet	41			15.5	0.3			60		265.2	0.25	92^{b}	89^{b}
Pooled SEM		-			-	-			-		-	-	0.7	0.9
p-	CF×P	-	<0.001	0.706	0.224	0.004	0.002	-	<0.001	-	0.258	0.067	0.075	
values	CF	-	<0.001	<0.001	0.004	<0.001	<0.001	-	<0.001	-	0.091	<0.001	<0.001	
	P	-	<0.001	0.590	0.303	<0.001	<0.001	-	<0.001	-	0.449	<0.001	<0.001	

a = rapidly degradable fraction [%]; *b* = potentially degradable fraction [%]; *c* = rate of degradation of *b* [h]; *lag* = lag time [h]; ED = effective degradation [%] of CP or ST at a passage rate of 0.05 h^{-1} (ED_5) and 0.08 h^{-1} (ED_8). Different superscripts within a column and main effect (or their interaction) indicate significant differences. *p*-values for main effects and their interactions are shown: pelleting (P: mash and pelleted) and compound feeds (CF: 1-8). Mash values in Table 5 are identical to observed values in Table 4, named respective to comparisons being made.

Table 6. Share of feed particles [% DM of feed sample] passing through sieves [diameter in mm] during the wet sieving procedure.

Feed		2.000		1.180		1.000		0.500		0.250		0.125		0.063	
		mean	*SD*	mean	*SD*	mean	*SD*	mean	*SD*	mean	*SD*	mean	*SD*	mean	*SD*
Maize		100	0.0	100	0.0	100	0.0	92	0.5	67	2.2	50	3.2	36	1.7
Wheat		100	0.0	100	0.0	100	0.2	94	0.6	82	1.7	74	1.9	65	3.0
Barley		100	0.0	100	0.1	99	0.3	90	0.6	72	0.6	62	1.2	55	0.8
Soybeans		100	0.0	100	0.1	100	0.1	97	0.3	84	0.3	74	0.8	67	0.3
Soybean meal		100	0.0	100	0.0	100	0.1	86	0.6	58	0.9	47	0.5	41	0.5
Rapeseed meal		100	0.0	100	0.0	100	0.0	95	0.8	60	1.7	39	1.0	29	0.2
Sunflower meal		100	0.0	100	0.0	100	0.1	89	0.8	53	0.6	34	1.1	26	0.2
Faba beans		100	0.0	100	0.1	100	0.3	89	0.7	74	1.2	70	1.2	67	1.1
DDGS		100	0.0	100	0.0	100	0.0	95	0.5	75	3.1	60	1.4	51	0.7
Maize gluten		100	0.0	100	0.1	100	0.1	92	0.5	69	2.0	57	1.3	50	0.4
Wheat bran		100	0.0	100	0.2	99	0.5	72	0.8	50	0.8	43	0.5	38	0.5
Sugar beet pulp		99	0.4	97	0.6	96	0.7	68	0.5	51	0.9	43	0.5	39	0.9
1	mash	100	0.0	100	0.0	100	0.1	92	0.2	71	0.7	58	0.9	48	0.6
	pellet	100	0.0	100	0.1	99	0.2	94	0.6	74	1.9	59	1.5	*	-
2	mash	100	0.0	100	0.0	100	0.1	89	0.4	68	0.4	56	0.3	47	0.4
	pellet	100	0.0	100	0.3	99	0.3	91	0.5	74	0.9	61	0.6	-	-
3	mash	100	0.0	100	0.0	99	0.2	87	0.8	65	1.8	55	1.2	49	2.0
	pellet	100	0.0	100	0.2	99	0.2	91	0.3	73	0.9	62	1.0	-	-
4	mash	100	0.0	100	0.0	100	0.1	91	0.2	71	0.6	62	3.6	54	3.1
	pellet	100	0.0	100	0.1	100	0.1	93	0.5	77	1.5	67	0.9	-	-
5	mash	100	0.0	100	0.0	99	0.2	92	0.5	67	0.4	51	0.6	41	0.4
	pellet	100	0.0	100	0.0	100	0.0	94	0.1	74	1.1	62	0.6	-	-
6	mash	100	0.0	100	0.0	99	0.2	89	0.6	66	0.5	53	0.5	46	0.5
	pellet	100	0.0	100	0.0	100	0.2	92	0.4	73	1.1	60	1.3	-	-
7	mash	100	0.0	100	0.0	99	0.1	88	0.2	67	0.2	55	0.2	47	0.2
	pellet	100	0.0	100	0.0	100	0.0	93	0.3	75	0.3	63	0.2	-	-
8	mash	100	0.0	100	0.3	100	0.3	88	0.4	67	0.6	57	1.1	51	0.6
	pellet	100	0.0	100	0.0	100	0.0	92	0.7	74	0.3	64	0.3	-	-

* Not determined.

Annex

Annex 1. Parameters describing the performance of NIRS-calibrations for N and starch (ST) concentration of single concentrate feeds, compound concentrate feeds, and their bag residues after *in situ* incubation

Parameter	N	ST
Calibration		
Units	% DM	% DM
SEL	0.4	1.4
N	674	588
Outliers	20	12
Min	0.5	0.5
Mean	2.49	31.6
Max	11.2	76.5
SD	1.50	23.3
SEC	0.10	1.82
R^2_C	>0.99	>0.99
SECV	0.12	2.02
R^2_{CV}	>0.99	>0.99
Number of terms	15	15
WL range/step	680-2500/1	680-2500/1
Pre-treatment (s)	2,8,8	1,8,8
Regression method	PLS	PLS
Validation		
N	214	206
Outliers	0	0
Min	0.7	0.5
Mean	2.76	29.1
Max	11.0	75.5
SD	1.74	23.6
R^2P	>0.99	>0.99
RMSEP	0.16	2.15
SEP(C)	0.16	2.15
Bias	<0.01	0.13
Intercept	<0.01	0.26
Slope	>0.99	>0.99
Global Distance Average	0.9	1.0
Nearest Neighbour Distance Average	0.2	0.2

SEL = standard error of laboratory; N = number of samples after outliers have been removed; Min, Mean, and Max = lowest, mean, and highest value of the reference dataset; SD = standard deviation of the reference values; SEC = standard error of calibration; R^2_C = R^2 of the calibration; SECV standard error of cross validation; R^2_{CV} = R^2 of the cross-validation; WL range/step = wavelength range and steps (nm); Pre-treatment (s) = pre-treatment of the spectra (deviation, gap, smooth); R^2P = R^2 of validation; RMSEP = root mean square error of the validation; SEP(C) = root mean square error of the validation corrected for bias; PLS = partial least square.

4.2. Manuscript 2

Ruminal fermentation characteristics and related feeding values of compound feeds and their constituting single feeds studied by using *in vitro* techniques

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animal

Ruminal fermentation characteristics and related feeding values of compound feeds and their constituting single feeds studied by using *in vitro* techniques

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Single concentrate feeds are mixed together forming compound feeds for cattle. However, knowledge regarding the potential interactions (associative effects) between the feeding values of single feeds in compound feeds is lacking. The main objective of the present study was to evaluate ruminal fermentation characteristics and feeding values of eight industrially produced compound feeds in mash form from their constituent single feeds for dairy cows through in vitro *assays. Additivity was given for gas production (**GP**), digestibility of organic matter (**dOM**) and utilisable CP at the duodenum (**uCP**). Additivity of CP fractions (determined using the Cornell Net Carbohydrate and Protein System (**CNCPS**)) was dependent on the fraction and compound feed type; however, the effective degradation calculated from CP fractions (ED_{CNCPS}) showed additivity. Additivity was not given for intestinal digestibility of rumen-undegraded protein (ID_{RUP}) for five out of eight compound feeds. Precise calculation of metabolisable energy (**ME**) of compound feeds from ME of single feeds was possible when using the same ME equations for all single and compound feeds. Compound feeds are often provided in pellet form; therefore, our second objective was to evaluate the effects of pelleting on ruminal fermentation characteristics and feeding values of compound feeds. Pelleting affected GP at 24 h (GP_{24}; up to 2.4 ml/200 mg DM), dOM (up to 2.3 percentage point (**pp**)) and ME (up to 0.3 MJ/kg DM), but these differences were overall small. More considerable effects of pelleting were observed for uCP, which was increased in all compound feeds except the two with the highest CP concentrations. The ID_{RUP} was lower in most compound feeds following pelleting (up to 15 pp). Pelleting also affected CP fractions in a non-systematic way. Overall, the effects of pelleting were not considerable, which could be because pelleting conditions were mild. Our third objective was to compare* in situ *ruminal CP degradation (ED_{IN_SITU}) of compound feeds with ED using two prediction methods based on CP fractions. ED_{IN_SITU} reference data were obtained from a companion study using the same feeds. Prediction accuracy of ED_{IN_SITU} and ED_{CNCPS} was variable and depended on the compound feed and prediction method. However, future studies are needed as to date not enough data are published to draw overall conclusions for the prediction of ED_{IN_SITU} from CP fractions.*

Keywords: additivity, associative effects, *in situ* prediction, mixed feed, interaction

Implications

Compound feeds are often fed to high-yielding dairy cows, both in mash and pellet form. Estimation of ruminal fermentation characteristics and feeding value of compound feeds from the single feeds contained therein is necessary for efficient feeding; therefore, this was assessed in the present study. Pelleting of compound feeds had only a negligible effect on ruminal fermentation characteristics and feeding values. Predictions of ruminal protein degradation based on CP fractions of the feed were not reliable.

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Introduction

Intensive dairy cow farming is reliant on adequate feeding to satisfy the increasing nutritive requirements of cows due to increasing milk yield. Concentrate compound feeds are often included in diets of dairy cows and are either provided with forages in the form of total mixed rations or separately. The additivity of feeding values of single feeds used in compound feeds is commonly assumed based on the presumption that no interactions between single feeds exist.

In vitro methods are widely used for feed evaluation because *in vivo* evaluations are expensive and laborious, and they require animals (GfE, 2017). To estimate the digestibility

of organic matter (**dOM**) and metabolisable energy (**ME**), measuring gas production (**GP**) by the Hohenheim gas test (**HGT**), as described by Menke and Steingass (1988), is an established assay. An extension of this method known as extended HGT (**eHGT**; Steingaß and Südekum, 2013) can be used to estimate the utilisable CP at the duodenum (**uCP**), which is the basis for the calculation of metabolisable protein used in the German protein evaluation system for cows (GfE, 2001). Calsamiglia and Stern (1995) developed a three-step method for estimating the intestinal digestibility of rumen-undegraded protein ($\mathbf{ID_{RUP}}$). These *in vitro* methods involve the use of ruminally fistulated animals as donors of rumen fluid. Sniffen *et al.* (1992) described a rapid CP fractionation method to be part of the Cornell Net Carbohydrate and Protein System (**CNCPS**). Therein, the CP in a feedstuff is separated into fractions by measuring N solubility. In an experiment by Chrenková *et al.* (2014), CP fractions were correlated with ruminal effective CP degradation (**ED**) values determined *in situ*. The CP fractions can be used to estimate ED values, which were found to correlate well with the ED values determined *in situ* (Shannak *et al.*, 2000). Additivity of feeding values of forages or mixes of forages and concentrates has been investigated utilising GP (Sandoval-Castro *et al.*, 2002; Robinson *et al.*, 2009; Niderkorn *et al.*, 2011) and uCP (Zhao *et al.*, 2005). However, to our knowledge, there has been no research on the additivity of ID_{RUP}. Also, comprehensive data on additivity of multiple single feeds in a compound feed are not available.

Compound feeds for cattle are often in pellet form, and pelleting can increase the availability of CP and starch (**ST**) or increase indigestible bonds, depending on the intensity of the pelleting process (Svihus and Zimonja, 2011). In compound feeds, these effects can depend on the choice of single feeds, and hence, they should be examined over a wide range of various compound feeds. The objective of the present study was to characterise GP and the related values of dOM and ME as well as uCP, ID_{RUP} and CP fractions of single feeds and the compound feeds produced with them, both in mash and pellet form. Three hypotheses were developed:

(I) Values of GP, dOM, ME, uCP, ID_{RUP} and CP fractions of compound feeds in mash form can be calculated from data obtained for single feeds;
(II) Pelleting significantly affects GP, dOM, ME, uCP, ID_{RUP} and CP fractions of compound feeds;
(III) Ruminal effective degradability of CP determined *in situ* can be predicted from CP fractions.

Material and methods

Samples of single and compound feeds

Eight compound feeds with different target CP concentrations (16%, 18%, 20%, 22%, 24%, 26%, 28% and 30% CP in DM) were mixed using 12 single feeds: maize, wheat, barley, soya beans, soya bean meal, rapeseed meal, sunflower meal, faba beans, dried distillers' grains with solubles (**DDGS**), maize gluten, wheat bran and sugar beet pulp. Between five and seven single feeds were included in each compound feed in different concentrations. Compound feeds were produced in mash and pellet form using standard industrial processes in the feed mill of RKW-Kehl (Kehl, Germany). Production and analysed nutrient concentrations and particle size distribution of all feeds were detailed previously (Grubješić *et al.*, 2019). Targeted CP concentrations were achieved in all compound feeds. Crude protein, ash, ether extract (**EE**), NDF assayed with a heat stable amylase and expressed exclusive of residual ash (**aNDFom**) and ADF expressed exclusive of residual ash (**ADFom**) did not differ more than one percentage point (**pp**) between calculated concentrations from single feeds and analysed concentrations in mash compound feeds.

Gas production kinetics, metabolisable energy and digestibility of organic matter

In vitro GP kinetics were measured using HGT following the procedure described by Seifried *et al.* (2016). Approximately 200 ± 5 mg of feed ground through a 1-mm sieve was transferred into graded glass syringes (100 ml volume). Fresh rumen fluid was obtained from two rumen-fistulated Jersey cows, one lactating and one not lactating. The lactating cow was provided a ration consisting of (on DM basis) 41.3% concentrate mix, 20.0% maize silage, 16.3% meadow hay, 15.0% grass silage, 3.6% barley straw, 2.4% mineral mix and 1.4% rapeseed meal. The other cow was provided a ration consisting of 35.4% maize silage, 35.4% grass silage, 24.6% meadow hay, 3.2% barley straw, 1.0% mineral mix and 0.4% urea. Cows had *ad libitum* access to feed.

The rumen fluid obtained from the two cows was mixed to a 1 : 1 ratio, filtered through two layers of cheesecloth, and a reduced buffer solution was added. Syringes were pre-warmed to 39°C before 30 ml of buffer-rumen fluid mix was poured into each syringe under constant CO_2 flow. Each feed was included in five separate HGT runs with two replicated syringes per feed in each run. Additionally, each run contained three syringes without feed samples (blanks) and three syringes with a concentrate standard feed. Cumulative GP was recorded after 2, 4, 6, 8, 12, 24, 48 and 72 h of incubation at 39°C under constant rotation. The following non-linear regression was fitted to the obtained GP data according to Seifried *et al.* (2016):

$$Y = b\text{GP} \cdot (1 - e^{-c\text{GP} \cdot 0.01 \cdot t}) \quad (1)$$

where *b*GP is the potential GP (ml/200 mg DM), *c*GP the rate of GP (%/h) and *t* the incubation time (h).

The dOM was calculated using GP at 24 h ($\mathbf{GP_{24}}$) corrected for the blanks and standard (GP_{24}; ml/200 mg DM) and chemical analysis according to Menke and Steingass (1988):

$$\text{dOM}\ (\%) = 9.0 + 0.9991\text{GP}_{24} + 0.0595\text{CP} + 0.0181\text{ash} \quad (2)$$

The ME was calculated using GP_{24} and specific to the type of feed, as follows:

(a) Maize, wheat, barley, faba beans, maize gluten and wheat bran according to Krieg *et al.* (2017):

$$\begin{aligned}\text{ME (MJ/kg DM)} = 0.9065 \cdot \\ (1.681 + 0.157\text{GP}_{24} + 0.0084\text{CP} + 0.0220\text{EE} - 0.0081\text{ash})\end{aligned} \quad (3)$$

(b) Non-cereal feeds (soya beans, soya bean meal, rapeseed meal, sunflower meal, DDGS and sugar beet pulp) according to Menke and Steingass (1988):

$$\begin{aligned}\text{ME (MJ/kg DM)} = 1.06 + 0.157\text{GP}_{24} + 0.0084\text{CP} \\ + 0.0220\text{EE} - 0.0081\text{ash}\end{aligned} \quad (4)$$

(c) Compound feeds according to GfE (2009):

$$\begin{aligned}\text{ME (MJ/kg DM)} = 7.17 - 0.01171\text{ash} + 0.00712\text{CP} \\ + 0.01657\text{EE} + 0.00200\text{ST} \\ + 0.00202\text{ADFom} + 0.06463\text{GP}_{24}\end{aligned} \quad (5)$$

In equations (2) to (5), CP, ST, EE, ash and ADFom are expressed in g/kg DM.

Utilisable CP at the duodenum

The eHGT method described by Steingaß and Südekum (2013) was used to estimate uCP and was conducted according to Westreicher-Kristen *et al.* (2015). Some former studies using this approach used the term 'modified HGT' and the abbreviation 'modHGT'. However, the term 'extended HGT' and the abbreviation 'eHGT' may be more appropriate as this method is not a real modification of the original HGT but an extension (measuring NH_3-N after incubation) and can be connected with GP_{24} measurement to estimate dOM and ME. Samples were incubated similarly to those in the HGT method described above. Donor cows had *ad libitum* access to a ration consisting of (on DM basis) 25.8% concentrate mix, 24.3% grass silage, 24.3% maize silage, 17.0% hay, 4.4% rapeseed meal, 2.2% barley straw and 2.0% mineral mix. Samples were incubated twice for different times (8 and 24 h), and a standard concentrate sample with known uCP concentration was included to check the variation of uCP results among runs. Each feed sample was incubated in five separate runs per incubation time. Following incubation, all syringes were rapidly frozen to minimise microbial fermentation. The following day, the NH_3-N concentration of incubation residues obtained from the syringes was analysed (Vapodest 50; C. Gerhardt GmbH & Co. KG, Königswinter, Germany). The NH_3-N concentration was used to estimate the uCP concentration as follows:

$$\begin{aligned}\text{uCP(g/kg DM)} = ((\text{NH}_3\text{-N}_{\text{blank}} + \text{N}_{\text{sample}} - \text{NH}_3\text{-N}_{\text{sample}}) \\ \cdot 6.25 \cdot 1000)/\text{weight}\end{aligned} \quad (6)$$

where N_{sample} is the amount of N from the feed sample (mg), NH_3-N_{sample} and NH_3-N_{blank} are the NH_3-N content of feed samples and blank incubation residues (mg) and weight is the weight of feed sample inserted into the glass syringe (mg DM). Effective uCP was estimated for theoretical ruminal passage rates (k) of 5 and 8%/h by plotting uCP values (y) against the natural logarithm of the incubation time (x) in a linear regression model and calculating the function values of ln (20) and ln (12.5), respectively (Steingaß and Südekum, 2013).

Intestinal digestibility of rumen-undegraded protein

The three-step enzymatic method of Calsamiglia and Stern (1995) was used to determine ID_{RUP}. Samples of single and compound feeds were ground through a 2-mm screen. The first step was a 16-h *in situ* incubation in the rumen, and this was conducted using three rumen-fistulated Jersey cows, following the procedure described in Seifried *et al.* (2016). A minimum of 60 mg of residual N per feed was accumulated for subsequent *in vitro* simulation of digestibility in the abomasum and duodenum. Two or three samples per feed containing 15 mg of residual N were incubated utilising 10 ml HCl (0.1 N, pH = 1.9), pepsin (1 g/l, Sigma P-7012; Sigma, St. Louis, MO, USA) and pancreatin solution (0.5 M KH_2PO_4 buffer standardised at pH 7.8 containing 50 ppm of thymol and 3 g/l of pancreatin, Sigma P-7545; Sigma). Trichloroacetic acid was added to stop enzymatic action and precipitate undigested proteins. Samples were centrifuged at 15 000 g for 25 min. Supernatants were analysed for soluble N by the Kjeldahl method (VDLUFA, 2007). Finally, ID_{RUP} was calculated as follows:

$$\text{ID}_{\text{RUP}}\ (\%) = (\text{N}_{\text{soluble}} / \text{N}_{\text{incubated}}) \cdot 100 \quad (7)$$

where $N_{soluble}$ is the amount of soluble N determined *in vitro* (mg) and $N_{incubated}$ is the total N that was incubated with pepsin and pancreatin (mg).

CP fractionation

Crude protein fractions were estimated according to the CNCPS (Sniffen *et al.*, 1992): fraction A represented the non-protein N, fraction B the true protein and containing three sub-fractions (B1 to B3) differing in their rate of ruminal degradation and fraction C the acid detergent insoluble N. To calculate CP fractions, non-protein N, buffer-soluble protein, neutral detergent insoluble N and acid detergent insoluble N were determined according to Licitra *et al.* (1996) for all samples of single and compound feeds. Table values of ruminal degradation rates of CP fractions of single feeds (Fox *et al.*, 2003) were used together with determined CP fractions to calculate ED_{CNCPS} using equation (8) (Fox *et al.*, 2003):

$$\begin{aligned}\text{ED}_{\text{CNCPS}}(\%\text{ of CP}) = \text{A} + \text{B1} \cdot ((\text{Prot-B1})/(\text{Prot-B1} + k)) \\ + \text{B2} \cdot ((\text{Prot-B2})/(\text{Prot-B2} + k)) \\ + \text{B3} \cdot ((\text{Prot-B3})/(\text{Prot-B3} + k))\end{aligned} \quad (8)$$

where ED_{CNCPS} is ED calculated from CP fractions, A and B1 to B3 are determined CP fractions, Prot-B1 to Prot-B3 are table values for ruminal degradation rates of CP fractions and *k* is the ruminal passage rate (5 or 8%/h). The ruminal degradation rate of faba beans was not reported in Fox *et al.* (2003), and thus, the value for lupins was used instead as they both belong to the legume family and show similar ED values (Goelema *et al*, 1998). Table values of ruminal degradation rates of compound feeds were not available; therefore, they were calculated from values of the respective single feeds. This calculation included weighting contributions of CP of single feeds to the total CP of the respective compound feed. Ruminal degradation rates were then used together with determined CP fractions to estimate the observed ED_{CNCPS} for mash and pelleted compound feeds.

An alternative prediction equation (Shannak *et al.*, 2000) based on CP fractions and NDF was used to estimate RUP of compound feed as follows:

$$\begin{aligned} &RUP_5 \text{ or } RUP_8(\text{g/kg CP}) = \beta_0 + \beta_1 \text{ CPPNDF} \\ &+ \beta_2(\text{CP} \times \text{B2}) + \beta_3(\text{CP} \times \text{C}) + \beta_4(\text{CP}(A + \text{B1})) \\ &+ \beta_5(\text{CP} \times \text{C}^2) + \beta_6(\text{PNDF} \times \text{B1}) \\ &+ \beta_7((\text{B3} + \text{C})\text{B2}) + e \end{aligned} \quad (9)$$

where RUP_5 or RUP_8 are RUP values for rumen outflow rates of $k = 5$ and 8%/h, respectively. The CPPNDF is the CP concentration in PNDF (NDF determined by manual filtration on paper) and all nutrients are given as g/kg DM, whereas A, B and C fractions are given as g/kg CP. Instead of CPPNDF and PNDF, the CP concentration in aNDF as well as aNDF was determined in the present study by the conventional method (VDLUFA, 2007). The general form of the equation is identical for RUP_5 and RUP_8, and the parameter estimated of β_0 to β_7 is given in Shannak *et al.* (2000). The ED_{CNCPS} was then calculated from RUP values for the given rumen outflow as:

$$ED_{CNCPS} \text{ (\% of CP)} = (1000 - \text{RUP})/10 \quad (10)$$

The ED_{CNCPS} values of compound feeds (calculated either with equation (8) or with equations (9) and (10)) were compared with measured ED_{IN_SITU} values of a companion study that determined *in situ* degradation values of the same feeds used in the present study (Grubješić *et al.*, 2019).

Additivity calculation

To evaluate the additivity of all traits of single feeds in a mash compound feed, the expected value of the compound feed was calculated based on weighted contribution of DM (for *b*GP, *c*GP, GP_{24}, dOM, ME and uCP) or CP (for ID_{RUP} and CP fractions) from single feeds to the DM and CP contained in the respective compound feed. These values are referred to as 'calculated' herein. To calculate the ME values of compound feeds from single feeds, two approaches were used. The ME values of single feeds were determined according to either equations (3) or (4) depending on the feed group or alternatively equation (5) for all single feeds.

Statistical analyses

Calculated and observed values of mash compound feeds, and values of mash and pellet compound feeds, were regressed using procedure REG (version 9.4 of SAS system for Windows SAS Institute, Cary, NC, USA). The REG procedure was also used to calculate if slopes and intercepts were significantly different from 1 and 0, respectively, by determination of 95% CI to detect possible associative effects.

Results

In vitro *ruminal fermentation and feeding values of single feeds*

Overall, ruminal fermentation characteristics and feeding values of single feeds varied widely (Table 1). The highest GP_{24} was found in maize (81 ml/200 mg DM) and the lowest in sunflower meal (36 ml/200 mg DM). The highest dOM was found in maize and wheat (96%) and the lowest in sunflower meal (65%). The highest ME was found in soya beans (16.0 MJ/kg DM) followed by maize (14.5 MJ/kg DM) and wheat (14.2 MJ/kg DM) and the lowest in sunflower meal (9.4 MJ/kg DM). The uCP concentration varied between 144 g/kg DM in wheat bran and 279 g/kg DM in soya bean meal for $k = 5$%/h, and between 158 g/kg DM in maize and wheat bran and 356 g/kg DM in soya bean meal for $k = 8$%/h. The ID_{RUP} ranged between 18% in wheat bran and 83% in maize and soya bean meal. All CP fractions varied widely between single feeds. This was reflected in the high variability of ED_{CNCPS} values.

Additivity of fermentation characteristics and feeding values

Calculated and observed ruminal fermentation characteristics and nutritional values of the mash compound feeds are presented in Table 2. Estimation of *b*GP, GP_{24} and dOM was precise as indicated by the slope of the regression lines (close to 1) and the high R^2 values (Table 3). Observed *c*GP differed numerically (0.3 to 0.7 pp) from calculated values, and the estimated slope of regression was only 0.68, associated with a large CI. Deviation of calculated and observed ME values was high when the specific equations for each group of feed were used. The comparison between calculated and observed ME showed a low R^2 value of 0.55 with a RMSE of 0.29 (Figure 1). However, when using the same ME equation (equation (5)) for all single and compound feeds, estimated ME of compound feeds from that of single feeds was precise, with an R^2 value of 0.99 (Figure 1).

Observed uCP was numerically lower than calculated in all compound feeds. However, the difference did not exceed 13 g/kg DM. The regression line slopes were close to 1 (0.97 for both $k = 5$ and 8%/h) and regression equations showed high R^2 values (0.88 and 0.96 for $k = 5$ and 8%/h, respectively).

Table 1. *In vitro* fermentation characteristics and feeding value of single feeds

	In vitro gas production			dOM	ME	uCP		ID_{RUP}	CP fractions				ED_{CNCPS}	
Single feed	*b*GP (ml/200 mg DM)	*c*GP (%/h)	GP_{24} (ml/200 mg DM)	(%)	(MJ/kg DM)	$k = 5$%/h (g/kg DM)	$k = 8$%/h (g/kg DM)	(%)	A (%)	B1 (%)	B2 (%)	B3 (%)	C (%)	$k = 5$%/h (%)	$k = 8$%/h (%)
Maize	92	5.7	81	96	14.5	163	158	83	12.5	5.9	66.6	14.9	0.0	65	55
Wheat	70	8.3	78	96	14.2	180	198	35	9.8	22.2	63.5	4.4	0.0	80	73
Barley	81	7.7	75	92	13.6	162	175	47	11.4	16.4	62.3	5.0	5.0	76	69
Soya beans	47	10.8	46	79	16.0	227	282	71	4.5	4.8	82.7	6.4	1.6	56	45
Soya bean meal	56	8.0	51	92	13.3	279	356	83	2.9	8.4	83.8	3.6	1.2	72	62
Rapeseed meal	50	8.5	45	78	11.3	241	280	26	7.1	13.4	62.5	10.2	6.8	73	65
Sunflower meal	39	8.9	36	65	9.4	166	205	46	8.3	27.3	54.6	5.9	3.9	76	69
Faba beans	70	8.3	63	90	12.9	202	224	32	12.7	42.3	38.7	4.2	2.1	80	75
DDGS	51	8.7	44	73	11.9	275	296	75	20.6	0.0	44.1	11.8	23.6	55	50
Maize gluten	62	6.8	54	78	11.0	188	196	59	48.5	6.2	33.8	8.6	2.9	79	75
Wheat bran	57	9.9	52	72	10.9	144	158	18	12.5	21.1	49.4	13.6	3.4	80	74
Sugar beet pulp	78	9.4	74	90	13.2	182	199	67	39.9	0.0	29.2	24.8	6.2	82	78

*b*GP = potential gas production; *c*GP = rate of gas production; GP_{24} = corrected gas production at 24 h; dOM = digestibility of organic matter; ME = metabolisable energy; uCP = utilisable CP for ruminal passage rates (k) of 5 and 8%/h; ID_{RUP} = intestinal digestibility of rumen-undegraded protein; CP fractions = crude protein fractions: A = non-protein nitrogen; B1 = rapidly degradable true protein; B2 = moderately degradable true protein; B3 = slowly degradable true protein, C = undegradable and indigestible true protein, determined using Cornell Net Carbohydrate and Protein System (CNCPS); ED_{CNCPS} = effective protein degradation for ruminal passage rates of 5 and 8%/h, calculated using Fox *et al.* (2003); DDGS = dried distillers' grains with solubles.

Additivity of single feeds to compound feeds

Observed ID_{RUP} in compound feeds differed from calculated values between 0 and 11 pp. Regression analysis between calculated and observed ID_{RUP} values showed an R^2 value of 0.64 and relatively large CIs for the slope and intercept, respectively.

Conformity between calculated and observed CP fractions depended on the specific fraction and the compound feed type. Confidence interval of the slope did not include the value of 1 only for B3 (CI = 1.22 to 2.18), even though the R^2 value for this parameter was high ($R^2 = 0.93$).

Results of the regression analysis of calculated and observed ED_{CNCPS} values showed small accuracy (R^2 of 0.36 and 0.43 for $k = 5$ and 8%/h, respectively). However, the slope values included 1 and intercept values included 0 and numerical differences were in most cases not even detectable (Table 2).

Effects of pelleting on ruminal fermentation characteristics and feeding value of compound feeds

Differences between mash and pellet compound feeds in GP_{24} did not exceed 3 ml/200 mg DM, 3 pp in dOM and 0.3 MJ ME/kg DM (Table 2). However, based on CI ranges (Table 3), the results indicated that pelleting did affect GP characteristics. The slopes and the intercepts for *b*GP, *c*GP, GP_{24}, dOM and ME were all significantly different from 1 to 0, respectively, even though the R^2 value was 0.93 or higher. Pelleting numerically increased uCP in compound feeds with lower CP concentration and decreased uCP in compound feeds with higher CP concentration. The slopes and intercepts were significantly different from 1 and 0, respectively, with considerable differences in R^2 values between $k = 5$%/h ($R^2 = 0.38$) and $k = 8$%/h ($R^2 = 0.83$). Pelleting decreased estimated ID_{RUP} in most compound feeds, with a maximum of 15 pp in compound feed 3. Pelleting increased estimated ID_{RUP} only in compound feed 1, but the difference was negligible (2 pp). Although the CI for the slope of ID_{RUP} included 1 and the R^2 value was high ($R^2 = 0.92$), the intercept was significantly different from 0. Pelleting did not systematically affect CP fractions in compound feeds (Table 3). Pelleting reduced the ED_{CNCPS} in most compound feeds slightly (up to 3 pp) for both $k = 5$ and 8%/h.

Prediction of in situ *ruminal CP degradation from CP fractions*

The ED_{CNCPS} values were smaller than ED_{IN_SITU} for all compound feeds, and the difference was up to 11 and 14 pp for $k = 5$ and 8%/h, respectively (Figure 2). Calculation of ED_{CNCPS} using individual CP fractions and tabular values for their specific degradation rates resulted in a very low variation from 71% to 77% ($k = 5$%/h) and 62% to 70% ($k = 8$%/h), whereas ED_{IN_SITU} of compound feeds showed wider variation from 74% to 88% ($k = 5$%/h) and 67% to 84% ($k = 8$%/h). The ED_{CNCPS} based on the regression analysis according to Shannak *et al.* (2000) resulted in a remarkable higher variability between compound feeds (from 67% to 95% for $k = 5$%/h and 61% to 86% for

Table 2. *In vitro* fermentation characteristics and feeding value of mash and pellet compound feeds and values calculated from single feeds

	Compound feed	*In vitro* gas production			dOM	uCP		ID_{RUP}	CP fractions					ED_{CNCPS}	
		*b*GP (ml/200 mg DM)	*c*GP (%/h)	GP_{24} (ml/200 mg DM)	(%)	$k = 5$%/h (g/kg DM)	$k = 8$%/h (g/kg DM)	(%)	A (%)	B1 (%)	B2 (%)	B3 (%)	C (%)	$k = 5$%/h (%)	$k = 8$%/h (%)
1	Calculated	77	7.0	73	92	181	193	63	12.9	12	66.3	7.9	0.9	75	67
	Mash	81	7.3	74	93	173	188	71	17.1	7.1	68.0	7.8	0.0	75	67
	Pellet	77	8.4	71	91	192	212	73	19.4	3.0	69.9	7.7	0.0	74	66
2	Calculated	69	7.9	66	87	190	205	46	16.5	11.8	54.5	10.8	6.4	76	69
	Mash	71	8.6	65	86	177	195	55	23.2	3.9	55.3	14.1	3.5	76	69
	Pellet	70	9.2	65	86	185	204	48	21.7	5.9	54.9	14.0	3.5	76	69
3	Calculated	68	8.2	62	84	175	193	44	17.3	21.3	51.3	6.8	3.2	77	70
	Mash	67	8.3	61	83	173	188	49	18.1	19.5	52.9	6.3	3.2	77	70
	Pellet	67	9.1	62	83	178	198	34	17.4	16.9	56.3	6.3	3.2	76	69
4	Calculated	72	8.2	66	89	192	216	59	9.3	15.3	65.3	6.0	4.1	71	62
	Mash	73	7.5	67	90	186	210	70	8.8	14.7	67.9	5.7	2.9	71	62
	Pellet	72	8.5	66	89	190	214	63	10.1	15.6	65.5	5.8	2.9	72	63
5	Calculated	68	7.6	63	87	200	222	49	9.3	18.6	59.8	7.7	4.6	73	66
	Mash	69	8.1	62	86	191	209	58	10.1	18.9	57.8	7.9	5.3	73	66
	Pellet	68	9.0	63	86	190	213	51	9.0	17.8	62.2	5.5	5.5	72	65
6	Calculated	63	8.7	58	83	196	225	49	9.3	19.0	61.1	7.3	3.3	73	66
	Mash	64	8.4	59	84	189	213	49	9.0	21.1	60.0	7.4	2.5	74	66
	Pellet	66	8.9	61	85	194	217	44	9.5	19.9	59.9	5.4	5.4	71	64
7	Calculated	55	8.9	54	81	199	233	50	9.3	14.1	66.4	7.1	3.0	73	65
	Mash	59	9.2	54	81	195	226	53	9.4	14.4	64.9	9.0	2.2	73	65
	Pellet	59	9.5	55	81	188	220	41	10.5	12.6	67.5	7.1	2.4	72	64
8	Calculated	62	8.7	57	85	203	240	60	7.1	17.9	67.6	5.0	2.4	73	65
	Mash	64	8.4	59	87	199	237	56	9.4	18.4	66.0	4.2	2.1	73	65
	Pellet	63	9.2	59	86	198	233	48	7.7	17.8	68.0	4.3	2.1	72	64

*b*GP = potential gas production; *c*GP = rate of gas production; GP_{24} = corrected gas production at 24 h; dOM = digestibility of organic matter; uCP = utilisable CP for ruminal passage rates (*k*) of 5 and 8%/h; ID_{RUP} = intestinal digestibility of rumen-undegraded protein; CP fractions = crude protein fractions: A = non-protein nitrogen; B1 = rapidly degradable true protein; B2 = moderately degradable true protein; B3 = slowly degradable true protein, C = undegradable and indigestible true protein, determined using Cornell Net Carbohydrate and Protein System (CNCPS); ED_{CNCPS} = Effective protein degradation for ruminal passage rates of 5 and 8%/h, calculated using Fox *et al.* (2003). Metabolisable energy (ME) values are presented in Figure 1.

Table 3. Results of simple linear regressions for *in vitro* fermentation characteristics and feeding values of compound feeds

	Calculated *v.* observed						Mash *v.* pelleted					
	Slope	Slope CI	Intercept	Intercept CI	R^2	RMSE	Slope	Slope CI	Intercept	Intercept CI	R^2	RMSE
In vitro gas production												
bGP	0.98	0.74 to 1.21	3.58	−11.95 to 19.11	0.95	1.70	0.79	0.64 to 0.93	13.80	3.87 to 23.72	0.97	1.06
*c*GP	0.68	0.03 to 1.33	2.67	−2.65 to 7.98	0.52	0.44	0.59	0.43 to 0.75	4.14	2.82 to 5.47	0.93	0.10
GP_{24}	0.96	0.78 to 1.14	2.72	−8.52 to 13.95	0.97	1.19	0.82	0.67 to 0.98	11.27	1.63 to 20.91	0.97	1.00
dOM	1.10	0.73 to 1.40	−4.95	−33.43 to 23.53	0.91	1.25	0.77	0.59 to 0.94	19.84	4.66 to 35.02	0.95	0.74
ME	–[1]	–	–	–	–	–	0.71	0.54 to 0.87	3.82	1.61 to 6.03	0.95	0.07
uCP												
$k = 5$%/h	0.97	0.61 to 1.33	−1.05	−70.00 to 67.89	0.88	3.73	0.37	−0.11 to 0.85	120.09	31.12 to 209.07	0.38	5.16
$k = 8$%/h	0.97	0.77 to 1.17	−1.26	−45.18 to 42.66	0.96	3.89	0.53	0.29 to 0.77	103.00	53.24 to 152.76	0.83	4.53
ID_{RUP}	0.95	0.23 to 1.66	7.86	−30.08 to 45.80	0.64	5.53	1.42	0.99 to 1.85	−31.27	−56.26 to −6.28	0.92	3.94
CP fractions												
A	1.37	0.79 to 1.94	−24.19	−92.26 to 43.88	0.85	23.09	0.95	0.69 to 1.20	7.15	−28.95 to 43.25	0.93	15.30
B1	1.65	0.94 to 2.35	−119.87	−237.17 to −2.56	0.84	26.55	0.94	0.64 to 1.24	−2.20	−49.96 to 45.55	0.91	20.23
B2	0.94	0.64 to 1.23	38.34	−144.37 to 221.05	0.91	19.22	0.88	0.52 to 1.24	88.96	−131.32 to 309.24	0.86	22.79
B3	1.70	1.22 to 2.18	−46.79	−82.89 to −10.69	0.93	8.71	0.94	0.57 to 1.32	−3.55	−34.68 to 27.58	0.86	12.03
C	0.71	0.13 to 1.29	2.36	−19.67 to 24.40	0.60	10.21	0.98	0.31 to 1.65	4.60	−15.84 to 25.04	0.68	10.83
ED_{CNCPS}												
$k = 5$%/h	1.24	−0.41 to 2.89	−12.35	−134.36 to 109.66	0.36	3.40	0.63	0.14 to 1.11	31.83	−7.02 to 70.68	0.62	2.08
$k = 8$%/h	1.41	−0.20 to 3.02	−20.20	−126.68 to 86.28	0.43	4.22	0.65	0.19 to 1.10	28.54	−4.71 to 61.79	0.67	2.55

*b*GP = potential gas production; *c*GP = rate of gas production; GP_{24} = corrected gas production at 24 h; dOM = digestibility of organic matter; ME = metabolisable energy; uCP = utilisable CP for ruminal passage rates (*k*) of 5 and 8%/h; ID_{RUP} = intestinal digestibility of rumen-undegraded protein; CP fractions = crude protein fractions: A = non-protein nitrogen; B1 = rapidly degradable true protein; B2 = moderately degradable true protein; B3 = slowly degradable true protein, C = undegradable and indigestible true protein, determined using Cornell Net Carbohydrate and Protein System (CNCPS); ED_{CNCPS} = effective protein degradation for ruminal passage rates of 5 and 8%/h, calculated using Fox *et al.* (2003). Observed values refer to values for compound feeds in mash form.

[1]Simple linear regressions of calculated and observed ME values are presented in Figure 1.

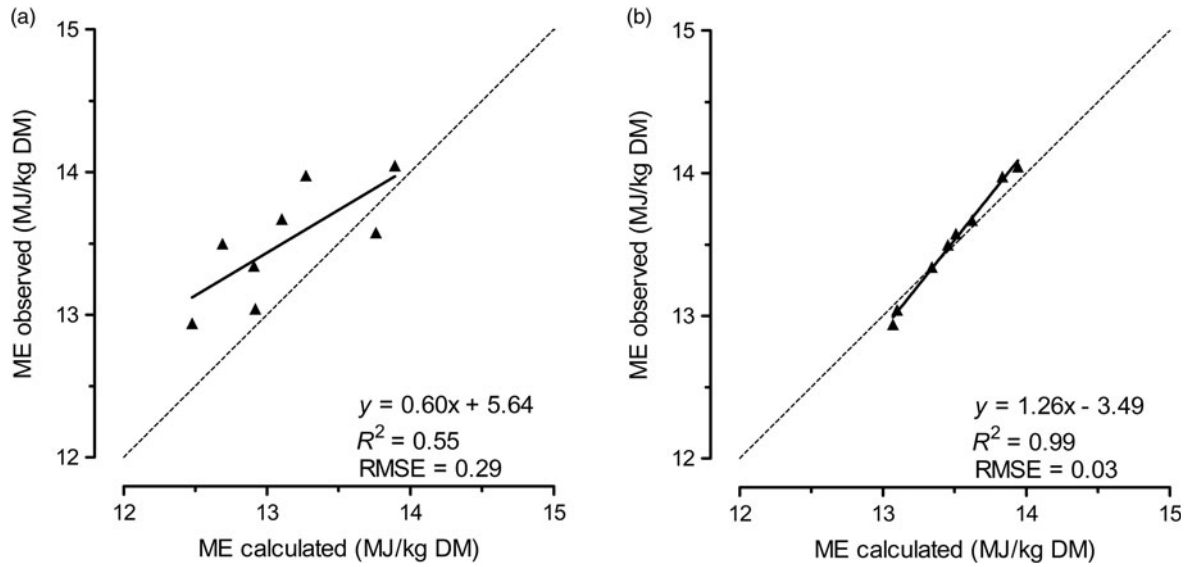

Figure 1. Comparison of calculated and observed metabolisable energy (ME) values of compound feeds using an *in vitro* ruminal fermentation technique. The ME values of compound feeds were calculated from ME values of single feeds that were determined according to the equations of: (a) Krieg *et al.* (2017) and Menke and Steingass (1988), respective of the feed group; or (b) GfE (2009) for all single feeds. The dotted line represents the angle bisector.

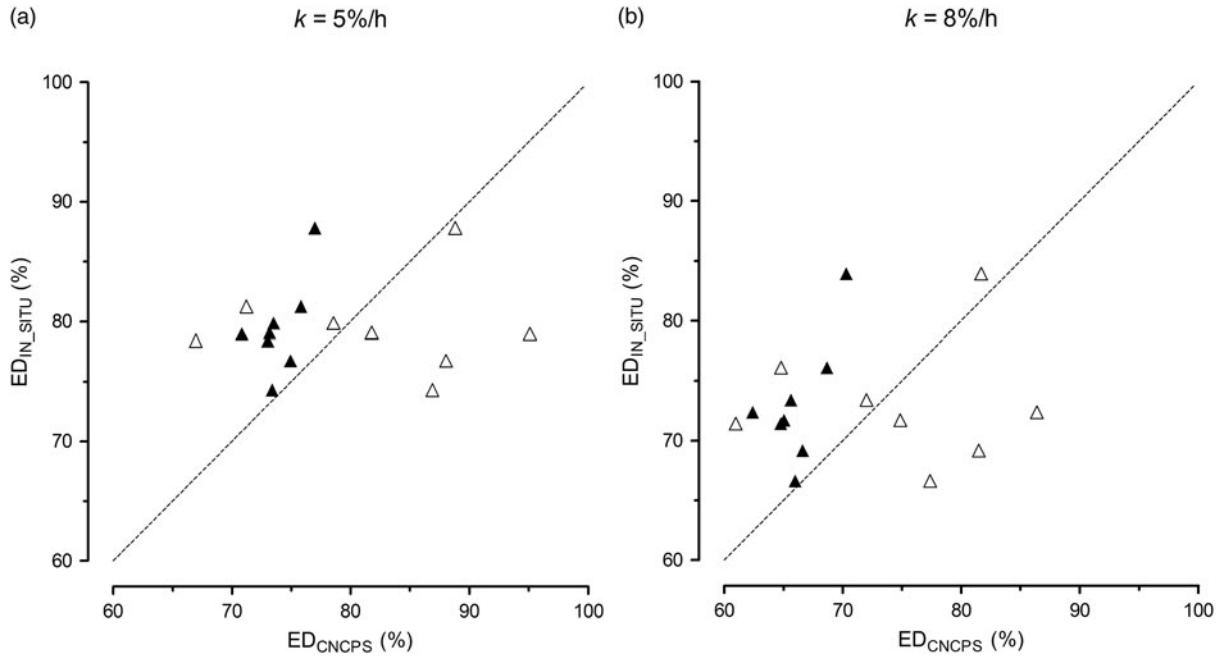

Figure 2. Comparison of ruminal effective protein degradation of compound feeds for ruminal passage rates of 5 and 8%/h based on CP fractions (ED_{CNCPS}) and calculated according to Fox *et al.* (2003) (▲) or Shannak *et al.* (2000) (△) and determined *in situ* (ED_{IN_SITU}; Grubješić *et al.*, 2019). The dotted line represents the angle bisector.

$k = 8\%/h$) compared to ED_{IN_SITU} and ranked feeds differently (Figure 2).

Discussion

Additivity of ruminal fermentation characteristics and feeding values

It was hypothesised that values of GP, dOM, ME, uCP, ID_{RUP} and CP fractions of compound feeds in mash form can be calculated from single feeds. Based on the results of the present study, this hypothesis can be accepted only in part. The agreement between calculated and observed values was good for *b*GP, *c*GP, GP_{24}, dOM and uCP, and thus, we consider these criteria to be additive. The highest deviation of calculated from observed value was 4 ml/200 mg DM for *b*GP, 0.7%/h for *c*GP, 2.3 ml/200 mg DM for GP_{24} and 2 pp for dOM.

For ME calculations, the equations chosen for single feeds largely affected the outcome of the comparison (Figure 1). In the literature, equations to predict ME from GP and nutrient

concentrations are often specific to single feeds or groups of feeds because this is associated with high prediction accuracy. However, it does not necessarily hold true across different feed groups and also for feed mixtures (Menke and Steingass, 1988). This caused issues when values for single feeds and related compound feeds were compared in the present study. We argue that the conclusion that additivity does not exist is misleading because it is an artefact of using different equations for different groups of feeds. When equation (5) was used to calculate the ME for all feeds (single and compound), the differences between calculated and observed ME became negligible (Figure 1).

Gas production techniques are underutilised for estimation of potential interactions between feeds (D'Mello, 2000) and energy evaluation, and previous research on additivity has most commonly focused on concentrate–forage mixes. Robinson *et al.* (2009) noted associative effects (15% to 25%) in mixture of alfalfa hay, barley grain, maize silage and soya bean meal using HGT in early phase of incubation, while they disappeared later. Similarly, Arhab *et al.* (2010) used mixtures of triticale and barley with a commercial concentrate supplement and found significant differences only in GP up to 8 h. In the present study, the discrepancy between calculated and observed GP at multiple time points can be considered to be negligible with a maximal difference of 2 ml/200 mg DM after 2 and 4 h of incubation. The observed GP_{24} was similar to the calculated ones and was considered to be additive. Since dOM and ME values are derived from GP_{24} (Menke and Steingass, 1988), this is an important finding and we presume that the differences between calculated and observed ME of compound feeds were caused only by the choice of ME equation. For the estimation of dOM values, the SD of regression residuals ($s_{y.x}$) is 3.07% (Menke and Steingass, 1988). The $s_{y.x}$ for estimation of ME values depends on the regression equation used, with a $s_{y.x}$ of 2.92 MJ/kg DM (Menke and Steingass, 1988) or a RMSE value of 1.98 MJ/kg DM (GfE, 2009) for equations (4) and (5), respectively. The RMSE between calculated and observed dOM and ME (equation (5)) of the compound feeds of the present study was 1.15% and 0.09 MJ/kg DM, respectively, and therefore markedly lower compared to the $s_{y.x}$ and the RMSE of the prediction equations. This underlines the assumption that additivity of those values is given.

The uCP consists of the RUP and microbial CP, as defined in the German feed protein evaluation system (GfE, 2001). Calculated uCP values corresponded well with observed values as the slope of regression and intercept was within their CI. A systematic overestimation of uCP was observed (up to 13 g/kg DM). Repeated measurements of observed uCP for each feed and incubation time were close together and showed low SD between runs up to 23 g/kg DM. However, also variation of observed and calculated uCP between compound feeds was low for both rumen outflow rates with a maximal differences of 49 g/kg DM. Due to the small variability between feeds and only eight data points for regression analysis, this systematical overestimation should be interpreted with caution and reference to their biological and practical relevance, which seems negligible. This contrasts with the findings of Zhao *et al.* (2005), who reported higher differences between calculated and observed uCP values in an experiment using 16 single feeds and 19 mixtures. They noted statistically significant and non-systematic differences between calculated and observed uCP values. However, the authors mentioned the possibility of incomplete incubation of some feeds due to incomplete mixing with the incubation liquid (Zhao *et al.*, 2005). Such an effect was avoided in the present study by the constant motion of the rotary incubator.

Calsamiglia and Stern (1995) highlighted the importance of ID_{RUP} for evaluating feed protein. GfE (2001) and NRC (2001) assumed a constant ID_{RUP} value of 80% for all feeds. In the CNCPS, ID_{RUP} was assumed to be 100% for CP fractions A, B1 and B2, 80% for fraction B3 and zero for fraction C (Fox *et al.*, 2003). A wide range of ID_{RUP} of single and compound feeds was found in the present study. Observed ID_{RUP} in most compound feeds was higher than calculated (up to 11 pp). Accurate estimation of ID_{RUP} from single feeds was thus not possible for all compound feeds of the present study using the three-step method. This is underlined by the analytical tolerance of the determination of ID_{RUP} which was set to maximal 10% relative deviation from the mean value otherwise the procedure was repeated. Relative deviation of replicates varied between 0.04 and 8.11% around the mean value for all feed samples of the present study. However, for five out of eight compound feeds, relative deviations of calculated ID_{RUP} from observed ID_{RUP} exceeded the value of 10%, which represents the analytical tolerance. This indicates that associative effects occurred when analysing ID_{RUP} of compound feeds. Those interactions between single feeds in mixture can occur in any of the three steps. Calculation of the 16 h *in situ* CP degradation of compound feeds from single feeds showed better additivity than ID_{RUP} with a slight tendency to overestimate CP degradation (1 to 7 pp). Compound feeds that showed higher deviations between calculated and observed *in situ* degradation also tended to show higher differences between the calculated and observed ID_{RUP}. Hence, associative effects seem more pronounced during the *in vitro* enzymatic part but play also a role in the first step of *in situ* incubation. As it seems that majority of the associative effects in the three-step method occurred during the *in vitro* enzymatic part, the results of the present study should be verified using the mobile bag technique as an alternative to the second step.

To our knowledge, CP fractions have not been previously studied for additivity. In the present study, observed CP fractions of mash compound feeds were often different from those calculated, as indicated by intercept values (for fractions B1 and B3 CI not including 0) and slopes (fraction B3 CI not including 1) and the wide CI range overall.

The accurate determination of CP fractions depends, among others, on accurate CP determination. For some CP fractions, differences between calculated and observed

values were higher than analytical tolerances for CP analysis (VDLUFA, 2019). This was the case for the A and B1 fraction of compound feeds 1 and 2, and the B3 fraction of compound feed 2. However, for all other CP fractions and compound feeds, the difference between the calculated and observed values of CP fractions is similar or even lower than the analytical tolerance of CP analysis. In addition, small variability between compound feeds (particularly for CP fraction C) probably lowered the accuracy of regression analysis. Consequently, interpretation of additivity for CP fractions is difficult from the results of the present study and different depending on the specific fraction and feed type. Additivity of ED_{CNCPS} was given for all compound feeds. However, the accuracy of regression analysis may be limited owing to the relatively small sample size ($n = 8$ compound feeds) of the present study. Therefore, we recommend to examine the additivity of CP fractions of single feeds in compound feeds in further experiments.

Effects of pelleting on ruminal fermentation characteristics and feeding value of compound feeds

The second hypothesis of the present study was that the pelleting process would significantly affect GP, dOM, ME, uCP, ID_{RUP} and CP fractions of compound feeds. Based on the present results, this hypothesis can be rejected. Even though the results of statistical analysis indicated an effect of pelleting on GP and related values of ME and dOM, uCP and ID_{RUP}, the overall numerical differences were negligible.

When heat is excessively applied during the processing of compound feeds, the intestinal digestibility of protein can be reduced owing to the formation of Maillard products which can neither be fermented nor digested (Sniffen *et al.*, 1992). Any optimum of processing conditions would aim to reduce CP degradability in the rumen without affecting ID_{RUP}. The data obtained *in situ* with the same feeds as used in the present study (Grubješić *et al.*, 2019) indicated that pelleting increased rumen degradation of some compound feeds, thus resulting in less RUP entering the small intestine. However, pelleting increased the share of smaller feed particles compared with the mash feeds, which might have increased the number of feed particles leaving the bags without microbial degradation, and thus overestimated degradation. This conclusion is consistent with the results of the present study. In the present study, pelleting increased uCP (which consists of RUP and microbial CP) of most compound feeds (16%, 18%, 20%, 22%, 24% and 26% of CP in DM) up to 24 g/kg DM. No difference was found in the two compound feeds with the highest CP concentrations (28% and 30% of CP in DM).

In a study using duodenally cannulated animals, Goelema *et al.* (1998) did not find an effect on intestinal protein digestibility of mixtures of lupins, peas and faba beans after toasting for 3 min at 132°C. This temperature was higher than the one applied in the present study (pelleting exit temperature of up to 80°C to 90°C). The process of toasting is however technologically not equal to pelleting, as factors other than heat (pressure and moisture) also differ and might result in chemical or physical changes of the substrate. In the present study, except for compound feed 1, ID_{RUP} decreased from 6 to 15 pp in all compound feeds by pelleting.

In situ incubations over 16 h were used to generate RUP for *in vitro* determination of ID_{RUP}, and results showed that degradation after 16 h increased between 1.4 and 6.4 pp in pelleted compound feeds compared to their corresponding mash feeds. It can therefore be assumed that RUP of mash feeds after *in situ* incubation contained more potentially digestible CP for the *in vitro* enzymatic steps to determine ID_{RUP}. This is underlined by the calculation of total tract digestibility (**TTD**) from the summation of 16 h *in situ* RUP and *in vitro* ID_{RUP} which showed that differences in TTD between mash and pelleted compound feeds ranged only between 0.2 and 2.2 pp and can therefore be considered to be negligible. The higher rumen-degraded protein of pelleted compound feeds might be attributed to a smaller particle size compared to mash feeds, as explained in the previous sections.

Pelleting did not have a large effect on CP fractions and ED_{CNCPS} values of compound feeds. Heat treatment during the pelleting process can denaturise protein fraction B2 making it insoluble, resulting in increased B2 and C fractions (Licitra *et al.*, 1996). Such an effect was not found in the present study, probably due to the temperature during pelleting not being very high.

Prediction of in situ *ruminal CP degradation from CP fractions*

The third hypothesis of the present study was that ED_{IN_SITU} could be predicted using CP fractions. Based on the present results, this hypothesis is rejected. Compared with the corresponding ED_{IN_SITU} data (Grubješić *et al.*, 2019), neither the calculation of ED_{CNCPS} using individual CP fractions and tabular values for their specific degradation rates (Fox *et al.*, 2003) nor ED_{CNCPS} using proximate nutrients and CP fractions based on regression analysis (Shannak *et al.*, 2000) showed adequate prediction accuracy for all compound feeds. However, for two (calculated according to Fox *et al.* (2003)) and three (calculated according to Shannak *et al.* (2000)) out of eight compound feeds, ED prediction with both methods was similar (differences ≤ 3 pp). Attempts of using CP fractions together with proximate nutrients to estimate *in situ* ruminal CP degradation of single and compound feeds showed varying success. Titze *et al.* (2018) reported an overestimation of ED_{CNCPS} of lupins using the approach of Fox *et al.* (2003), for an average of 10 pp. In the present study, ED_{CNCPS} was generally lower than ED_{IN_SITU} for all compound feeds and prediction accuracy was very variable with differences from 1 to 14 pp. A problem when using the approach of Fox *et al.* (2003) is the necessity of using tabulated values for the degradation rate of the specific CP fractions. It was not mentioned how degradation rates were obtained, how many samples the provided mean values are based on and how high the range of degradation rates for individual CP fractions of the same feedstuff was. Shannak *et al.* (2000)

derived their prediction equations from selected proximate nutrients and CP fractions for *in situ* RUP values including 11 dairy compound feeds. Therefore, prediction of ED of compound feeds may be possible with good accuracy. Shannak *et al.* (2000) found differences between *in situ* RUP values and respective estimates of up to 79 g/kg CP; however, 8 out of 11 RUP values had differences $\leq$ 50 g/kg CP. For samples of the present study, ED_{IN_SITU} and ED_{CNCPS} calculated according to Shannak *et al.* (2000) differed by up to 16 pp and hence 5 out of 8 compound feeds had differences between estimated and *in situ* RUP $\geq$ 100 g/kg CP for $k = 5$ and 8%/h. Poor estimation may result from differences in the assay details because NDF was determined by manual filtration in the study of Shannak *et al.* (2000), and authors stated that results may deviate from those obtained with the conventional NDF method which was used in the present study. Moreover, NDF values ranged between 212 and 554 g/kg DM in the 11 compound feeds of Shannak *et al.* (2000) and only between 142 and 255 g/kg DM in the present study. Shannak *et al.* (2000) also included forages and special by-products in the development of the regression equations, which is another difference to the present study. It is therefore recommended to extend the existing database. More accurate equations may be developed when covering a wider range of feedstuff groups.

Conclusion

We conclude that, when formulating compound feeds for cattle, single feed data for GP_{24}, dOM, ME and uCP are additive, while those for ID_{RUP} are not. Additivity of CP fractions is dependent on the fraction and compound feed type, whereas ED_{CNCPS} is precisely additive. The pelleting process had little effect on ruminal fermentation characteristics and feeding values of compound feeds, probably because heat exposure was moderate. Using CP fractions in the present study did not reliably predict *in situ* ruminal CP degradation of compound feeds: more studies are needed to extend the database for the development of prediction equations.

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Declaration of interest

No potential conflict of interest is reported by the authors.

Ethics statement

The use of rumen-cannulated cows for rumen fluid collection was approved by the Regierungspräsidium Stuttgart, Germany, approval number A401-14 TE.

Software and data repository resources

No data were deposited in an official repository.

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4.3. Manuscript 3

Determination of *in situ* ruminal degradation of phytate phosphorus from single and compound feeds in dairy cows using chemical analysis and near-infrared spectroscopy

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Determination of *in situ* ruminal degradation of phytate phosphorus from single and compound feeds in dairy cows using chemical analysis and near-infrared spectroscopy

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*The ruminal degradation of P bound in phytate (**InsP$_6$**) can vary between feeds, but data on ruminal degradation of InsP$_6$ from different feedstuffs for cattle are rare. One objective of this study was to increase the data base on ruminal effective degradation of InsP$_6$ (**InsP$_6$ED**) and to assess if InsP$_6$ED of compound feeds (**CF**) can be calculated from comprising single feeds. As a second objective, use of near-infrared spectroscopy (**NIRS**) to predict InsP$_6$ concentrations was tested. Nine single feeds (maize, wheat, barley, faba beans, soybeans, soybean meal (**SBM**), rapeseed meal (**RSM**), sunflower meal (**SFM**), dried distillers' grains with solubles (**DDGS**)) and two CF (**CF1/CF2**), consisting of different amounts of the examined single feeds, were incubated for 2, 4, 8, 16, 24, 48 and 72 h in the rumen of three ruminally fistulated Jersey cows. Samples of CF were examined before (**CF1/CF2 Mash**) and after pelleting (**CF1/CF2 Pellet**), and InsP$_6$ED was calculated for all feeds at two passage rates (**InsP$_6$ED$_5$**: k = 5%/h; **InsP$_6$ED$_8$**: k = 8%/h). For CF1 and CF2, InsP$_6$ED was also calculated from values of the respective single feeds. Near-infrared spectra were recorded in duplicate and used to establish calibrations to predict InsP$_6$ concentration. Besides a global calibration, also local calibrations were evaluated by separating samples into different data sets based on their origin. The InsP$_6$ED$_8$ was highest for faba beans (91%), followed by maize (90%), DDGS (89%), soybeans (85%), wheat (76%) and barley (74%). Lower values were determined for oilseed meals (48% RSM, 65% SFM, 66% SBM). Calculating InsP$_6$ED of CF from values of single feeds underestimated observed values up to 11 percentage points. The NIRS calibrations in general showed a good performance, but statistical key data suggest that local calibrations should be established. The wide variation of InsP$_6$ED between feeds indicates that the ruminal availability of P bound in InsP$_6$ should be evaluated individually for feeds. This requires further* in situ *studies with high amounts of samples for InsP$_6$ analysis. Near-infrared spectroscopy has the potential to simplify the analytical step of InsP$_6$ in the future, but the calibrations need to be expanded.*

Keywords: feed evaluation, phosphorus availability, phytate degradation, rumen, analytical method

Implications

Phosphorus is essential for health, milk production and reproduction of dairy cows but contributes to environmental pollution when excreted. In plant seeds, P is mainly stored as phytate, but phytate degradation and, thus, availability of P in the rumen vary widely between different feeds. Data on ruminal phytate degradation of feeds commonly fed to dairy cows improves diet calculations contributing to an adequate P supply of the animals. In the future, the data base on ruminal phytate degradation can be further increased when near-infrared spectroscopy is used to predict phytate concentrations instead of elaborate chemical analysis.

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Introduction

An adequate supply of P is essential to ensure health and performance of dairy cows. However, faecal P excretion increases with P intake in a linear manner (Wu *et al.*, 2001), and P concentrations in the diet exceeding the animals' requirement lead to increased faecal P excretion. Phosphorus losses can contribute to eutrophication of natural waters (Desmit *et al.*, 2018) and, thus, excessive P supply in animal nutrition has to be avoided.

In plant seeds and by-products, P is contained predominantly as phytate (any salt of phytic acid; *myo*-inositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate); **InsP$_6$**). Rumen microorganisms show substantial phytase activity (Yanke *et al.*, 1998) which enables the hydrolytic cleavage of

P bound in $InsP_6$ (**$InsP_6$-P**) and subsequent P absorption in the intestine. However, results of studies examining total tract disappearance of $InsP_6$ are inconsistent. While several studies found only low faecal $InsP_6$ excretion of about 5% of ingested $InsP_6$ (e.g. Morse *et al.*, 1992; Ray *et al.*, 2013), others reported higher proportions of $InsP_6$ excreted (e.g. Haese *et al.*, 2014: up to 15%; Kincaid *et al.*, 2005: more than 20% of ingested $InsP_6$). Some of the observed differences can likely be explained by the wide variation of feed ingredients used in the diets. Earlier *in vitro* and *in situ* studies have shown that progression and extent of ruminal $InsP_6$ disappearance differ between feedstuffs. In rapeseed meal (**RSM**), $InsP_6$ disappearance proceeded slowly compared to maize (Haese *et al.*, 2017a), soybean meal (**SBM**) and wheat (Haese *et al.*, 2017b), leading to a lower effective $InsP_6$ degradation of RSM in the rumen compared to SBM (Park *et al.*, 1999). However, data on effective degradation of $InsP_6$ (**$InsP_6$ED**) in common feeds for cattle are rare to date. Thus, the first objective of the present study was to determine $InsP_6$ED from different single feeds used in cattle feeding. Furthermore, we determined $InsP_6$ED of compound feeds (**CF**) to assess if $InsP_6$ degradation values from single feeds are additive in CF. This would allow for calculations of $InsP_6$ED for any compound feed if respective values are given for the utilised single feeds. Increased data on the ruminal availability of $InsP_6$-P from different feeds may allow for more precise calculation of dietary P supply of dairy cows in the future.

In situ studies to determine $InsP_6$ED provide a large number of samples to be analysed for inositol phosphates (**InsPs**). Most commonly, high-performance ion chromatography (**HPIC**) with gradient elution or similar chromatography is used to separate InsPs and their isomers in feeds (Blaabjerg *et al.*, 2010). However, this technique is laborious and costly and is not established as a routine method for common feed analysis. Hence, faster and easier methods for analysis of $InsP_6$ would be beneficial to increase the data base of ruminal $InsP_6$ degradation of feeds. Various studies showed that near-infrared spectroscopy (**NIRS**) can be used to predict the concentration of $InsP_6$ (Zhao *et al.*, 2017) and $InsP_6$-P (Tahir *et al.*, 2012; Aureli *et al.*, 2017), while studies that applied this technique to *in situ* samples were not reported. However, for cereal grains, NIRS has been successfully used to predict CP and starch in bag residues after ruminal incubation (Krieg *et al.*, 2018a). Hence, the second objective of this study was to establish calibrations to predict the $InsP_6$ concentration of feeds and ruminally incubated bag residues using NIRS. In order to examine the suitability of NIRS estimations for the usage in *in situ* studies, $InsP_6$ED calculated from NIRS-derived $InsP_6$ concentrations was compared to those calculated from chemically analysed $InsP_6$ concentrations of the samples.

Material and methods

Samples and incubations

Samples of single and compound feeds and their respective bag residues originated from an *in situ* study described in detail by Grubješić *et al.* (2019). Nine single feeds (maize, wheat, barley, faba beans, soybeans, SBM, RSM, sunflower meal (**SFM**), dried distillers' grains with solubles (**DDGS**)) and two CF (**CF1, CF2**) composed of different amounts of these single feeds were used for analysis of InsPs. Compound feed 1 consisted of 10% maize, 46% barley, 16% faba beans, 18% soybeans, 5% SBM and 5% DDGS, while CF2 contained 32% maize, 12% wheat, 16% faba beans, 8% SBM, 17% RSM, 10% SFM and 5% DDGS (values on DM basis). The CF were produced in a commercial feed mill as described in detail by Grubješić *et al.* (2019). In brief, single feeds were ground through a 3 mm sieve and mixed into the CF. Subsequently, one portion of the compound feed was pelleted at 50°C to 60°C (exit temperature 80°C to 90°C). For the *in situ* incubations of CF1 and CF2, samples were taken before (**Mash**) and after pelleting (**Pellet**).

The ruminal incubation followed the procedure of Seifried *et al.* (2017) and was also described in detail by Grubješić *et al.* (2019). In brief, feed samples were ground to pass a 2 mm sieve and 8 g were weighed into polyester bags (10×20 cm, pore size 50 μm, ANKOM Technology, USA) with 3 to 5 replicates per sample, incubation time and animal. The bags were incubated in the rumen of three rumen-fistulated Jersey cows for 2, 4, 8, 16, 24, 48 and 72 h and washed in a washing machine after incubation. Values for incubation time 0 h were gained by washing three replicates of each feed sample in the washing machine without ruminal incubation. For analysis, the dried replicates were weighed and pooled per feed sample, incubation time and animal.

Chemical analysis

Dry matter of feed samples and bag residues was analysed according to the official methods used in Germany (Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten, 2007). Analysis of $InsP_6$ and isomers of lower InsPs (myo-inositol pentakisphosphate (**$InsP_5$**), myo-inositol tetrakisphosphate (**$InsP_4$**) and myo-inositol trisphosphate (**$InsP_3$**)) was performed as described by Zeller et al. (2015) with slight modifications regarding sample size and agent used for extraction. In brief, 0.1 g of the sample was extracted for 30 min with 1.0 ml of an extracting agent (0.2 Mol ethylenediaminetetraacetate and 0.1 Mol NaF, pH 8.0) on a rotary shaker. After centrifugation, the supernatant was removed, preserved on ice and the residue re-suspended with 0.5 ml extracting agent and extracted again for 30 min. The supernatants of both extraction steps were merged, filtered and centrifuged. Filtrates were analysed by HPIC (ICS-3000, Fa. Dionex, Idstein, Germany) and UV detection at 290 nm.

Calculations

For each feed, degradation parameters ***a*** (%; rapidly disappearing fraction), ***b*** (%; potentially degradable fraction), ***a*** + ***b*** (%; maximum degradation/plateau) and ***c*** (%/h; degradation rate) of $InsP_6$ were calculated based on HPIC-derived

$InsP_6$ concentrations using the equations described by Orskov and McDonald (1979) (equation (1)) and McDonald (1981) (equation (2)).

$$\text{Deg} = a + b \times (1 - e^{-ct}) \quad (1)$$

$$\text{Deg} = a + b \times \left(1 - e^{-c(t-L)}\right) \quad \text{for } t > L \quad (2)$$

where Deg (%) is the ruminal degradation of $InsP_6$ after t h and L represents lag time. Using the GraphPad Prism software (Version 5.0 for Windows, GraphPad Software, CA, USA), the best fitting model for each feed was selected based on the Akaike Information Criterion. For estimation of degradation values, estimations of fraction a and fraction $a + b$ were constrained to 0 and 100%, respectively. The degradation parameters of $InsP_6$ were then used to calculate the $InsP_6$ED at ruminal outflow rates of $k = 5$ (**$InsP_6ED_5$**) or 8 (**$InsP_6ED_8$**) %/h with either

$$InsP_6ED = a + [(b \times c)/(c + k)] \quad (3)$$

according to Orskov and McDonald (1979) or

$$InsP_6ED = a + [(b \times c)/(c + k)]\ e^{-kL} \quad (4)$$

according to Wulf and Südekum (2005).

For the CF, the degradation parameters and $InsP_6$ED values were additionally calculated from the observed values of single feeds as described by Grubješić *et al.* (2019) using

$$dCF_{(1,2)}calc = [(dSF_1 \times w_1) + (dSF_2 \times w_2) + \cdots + (dSF_i \times w_i)]/100 \quad (5)$$

$dCF_{(1,2)}$calc = calculated degradation characteristics (a, b, c, lag, $InsP_6ED_5$, $InsP_6ED_8$) of CF1 or CF2
dSF_i = observed degradation characteristics (a, b, c, lag, $InsP_6ED_5$, $InsP_6ED_8$) of single feed i
w_i = weighted $InsP_6$ contribution of single feed i to total $InsP_6$ pool of CF1 or CF2

Degradation parameters and $InsP_6$ED were calculated for each cow separately, using cow as experimental unit in statistical analysis.

Near-infrared spectroscopy

Because the number of feeds used in this study was relatively low for developing NIRS calibrations for $InsP_6$, values of samples from earlier *in situ* studies were added to the data pool. All additional data originated from studies where different feeds were ruminally incubated and analysed for $InsP_6$ concentrations using HPIC as described before. The additional data included values for barley, maize, rye, triticale and wheat (Seifried *et al.*, 2016 and 2017; Krieg *et al.*, 2017) and four RSM samples (Haese *et al.*, 2017c). Different combinations of samples were tested for the establishment of calibrations in order to compare the performance of local calibrations (including only one type of feed, e.g. cereal grains) with global calibrations (including all feed types) and to achieve the overall best performance. A total of seven data sets was created using different combinations of feeds and corresponding bag residues:

Data set 1: all values for feeds and bag residues of the present study
Data set 2: all values for feeds and bag residues of the present study and the additional studies (Seifried *et al.*, 2016 and 2017; Haese *et al.*, 2017c; Krieg *et al.*, 2017)
Data set 3: data set 2, but excluding all values for rye and triticale
Data set 4: only values for feeds and bag residues from grain samples of the present study and the additional studies
Data set 5: data set 2, but excluding all values for grain samples
Data set 6: data set 2, but excluding all values for CF
Data set 7: data set 2, but excluding all values for CF and grain samples.

Number of samples used for calibration and validation data sets are shown in Table 1.

Spectra were recorded in duplicate from 680 to 2500 nm (SpectraStar 2500X, Software: Unity InfoStar Version 3.11.1, Unity Scientific, Brookfield, CT). Additionally, spectra of an internal standard as well as external standards (US-STDS-0001 – STD, Wavelength cert, R99 and US-STDS-0003 – STD, Wavelength cert, R99/Poly; Unity Scientific, Brookfield, CT) were recorded throughout the measurements. Mathematical treatment of the spectra and calibrations computation were carried out using the software Ucalibrate (Version: 3.0.0.23; Unity Scientific, Brookfield, CT). The spectra were averaged per sample, and the averaged spectrum of each sample was mathematically pre-treated by standard normal variates

Table 1 *Number (*n*) of feed samples used for calibration development and validation. Mean and range of chemically analysed phytate ($InsP_6$) concentration of feeds and bag residues after* in situ *incubation*`

	Calibration				Validation			
		Mean	Min	Max		Mean	Min	Max
	n	(μmol/g DM)			*n*	(μmol/g DM)		
All[1]	259	18.1	1.3	66.5	102	18.4	1.3	65.2
Maize[1,2]	24	8.8	1.3	16.6	10	8.7	1.6	15.4
Wheat[1,3]	24	15.0	1.9	43.9	9	16.1	2.9	41.0
Barley[1,4]	25	14.6	2.1	28.8	9	14.2	2.8	21.4
Faba beans[1]	10	10.0	2.1	21.7	4	9.2	1.3	16.6
Soybeans[1]	10	13.6	2.8	21.9	4	13.6	3.7	18.6
Soybean meal[1]	9	10.7	31.6	25.0	4	10.9	31.4	23.7
Rapeseed meal[1,5]	69	31.0	1.3	66.5	27	30.2	1.6	65.2
Sunflower meal[1]	9	7.1	63.5	39.3	5	7.1	63.3	42.8
DDGS[1]	10	3.3	1.5	7.0	4	3.0	1.6	1.6
CF1, CF2 Mash[1]	20	13.9	2.1	25.3	8	13.7	2.3	25.0
CF1, CF2 Pellet[1]	19	11.5	2.2	20.4	8	10.9	2.3	20.3
Rye[4]	15	8.0	6.6	9.8	5	8.1	6.7	9.2
Triticale[4]	15	10.1	8.5	13.6	5	9.9	8.6	11.0

Min = minimum value; Max = maximum value.
Samples of [1]the present study, [2]Seifried *et al.* (2016) [3]Seifried *et al.* (2017) [4] Krieg *et al.* (2017), [5]Haese *et al.* (2017c).
DDGS = dried distillers' grains with solubles; CF1 = compound feed 1 (containing 10% maize, 46% barley, 16% faba beans, 18% soybeans, 5% soybean meal, 5% DDGS on DM basis); CF2 = compound feed 2 (containing 32% maize, 12% wheat, 16% faba beans, 8% soybean meal, 17% rapeseed meal, 10% sunflower meal, 5% DDGS on DM basis).

and detrending. Derivations of the spectra were computed using a derivation gap and smoothing steps of eight. The derivation option varied between no derivation and first- or second-order derivation. Subsequently, the spectra were used for calibration calculation. The samples were split into a calibration and a validation set for each feed type as outlined in Table 1, attempting to include the whole range of $InsP_6$ concentrations in both calibration and validation sets.

Three wavelength segments were compared: (1) the complete recorded spectrum (680–2500 nm), (2) the recorded spectrum constricted for 50 nm from the beginning and the end (730 to 2450 nm) and (3) the segment of 1250 to 2450 nm. Segment 2 was used to eliminate possible drifts near the limit of the detection. Segment 3 was used because most N–H and C–H bonds are known to be located in this area and because the protein and $InsP_6$ concentration correlated in RSM and SBM after ruminal *in situ* incubation (Haese *et al.*, 2017b). Each of the three wavelength segments was combined with each derivation, resulting in nine calibrations per data set. Stepwise forward partial least squares (PLS)-regression was used to compute calibrations. Number of groups for cross validation (**CV**) varied, depending on the number of samples in the calibrations. The T-limit for outlier detection was set to 2.5 (predicted *v.* reference value), and global distance limit was set to 13.

Calibration evaluation was carried out using the standard error of calibration (**SEC**) and the standard error of prediction (**SEP**) as a measure for the accuracy of the calibration (Bellon-Maurel *et al.*, 2010). Coefficients of determination (predicted *v.* reference) were also considered. The performance of the calibrations was further evaluated using the bias, the intercept and the slope of the validation step. The target values were zero for the bias and the intercept and one for the slope.

To evaluate the suitability of NIRS as alternative method to HPIC in *in situ* experiments, $InsP_6$ED was additionally calculated based on NIRS predicted $InsP_6$ concentrations of the feeds and bag residues according to equations 1 to 4 (**$InsP_6$ED NIRS**). The $InsP_6$ concentrations were predicted using the most accurate calibration and data set. These $InsP_6$ED values were then compared to $InsP_6$ED values deduced from $InsP_6$ concentrations measured using HPIC (**$InsP_6$ED HPIC**).

Statistical analysis

Degradation parameters *a*, *b*, *c* and lag as well as $InsP_6$ED values were statistically analysed with the SAS MIXED procedure (SAS System for Windows, Version 9.4, SAS Institute, Cary, NC, USA). For single feeds, a one-factorial approach with the following model was used:

$$Y_{ij} = \mu + A_i + \mathrm{SF}_j + e_{ij}$$

with Y_{ij} as responsive mean, μ as overall mean, A_i as random effect of animal ($i = 1, 2, 3$), SF_j as fixed effect of single feed (j = maize, wheat, barley, faba beans, soybeans, SBM, RSM, SFM, DDGS) and e_{ij} as residual error.

Compound feeds were analysed in a two-factorial approach with the model:

$$Y_{ij} = \mu + A_i + \mathrm{CF}_j + T_k + \mathrm{CF}_j\mathrm{T}_k + e_{ijk}$$

where CF_j is the fixed effect of compound feed (j = CF1, CF2), T_k is the fixed effect of type (k = Mash, Pellet, Calculated), and CF_jT_k is the interaction of CF_j and T_k. Data are presented as least-squares means (LS means) and pooled standard error of the means (pooled SEM).

For comparison of the $InsP_6$ED values based on chemical and NIRS derived $InsP_6$ concentrations also a two-factorial approach was used:

$$y_{i,j,k} = \mu + A_i + M_j + F_k + M_jF_k + e_{ijk}$$

where M_j is the method used to determine $InsP_6$ concentration (j = HPIC, NIRS), F_k the feed (k = maize, wheat, barley, faba beans, soybeans, SBM, RSM, SFM, DDGS, CF1 Mash, CF2 Mash, CF1 Pellet, CF2 Pellet), and M_jF_k is the interaction of M_j and F_k.

Statistical significance was declared at $P < 0.05$ for all models. Following a significant *F* value, *t*-tests were performed to show individual significant differences between means.

Results

Concentrations of inositol phosphates in single and compound feeds

The concentration of $InsP_6$ varied from 7.0 μmol/g DM (4.6 g/kg DM) to 49.9 μmol/g DM (32.9 g/kg DM) between the examined feeds (Table 2), with the lowest $InsP_6$ concentrations in DDGS and cereal grains (7.0 to 12.4 μmol/g DM; 4.6 to 8.2 g/kg DM) and the highest in RSM and SFM (36.5 and 49.9 μmol/g DM; 24.1 and 32.9 g/kg DM, respectively). The $InsP_6$ concentrations in CF1 (Mash and Pellet) were considerably lower compared to CF2.

In cereal grains, only traces of $InsP_5$ were determined (below limit of quantification, approximately 0,3 μmol/g DM). In the other feeds, $InsP_5$ concentrations ranged from 1.5 μmol/g DM to 7.5 μmol/g DM (Table 2). The highest $InsP_5$ concentrations were determined in RSM and SFM (5.4 and 7.5 μmol/g DM, respectively). Concentrations of InsPs lower than $InsP_5$ overall were very low and only for DDGS slightly above the quantification limit (1.4 μmol/g DM $InsP_4$ and 1.5 μmol/g DM $InsP_3$, data not shown).

Degradation parameters and effective degradation of phytate from single feeds

Ruminal degradation parameters *a*, *b* and *c* differed significantly between the single feeds and ranged from 0% (RSM) to 77% (DDGS) for fraction *a*, from 22% (DDGS) to 100% (RSM) for fraction *b* and from 7.3%/h (RSM) to 28.2%/h (SFM) for degradation rate *c* (Table 3). The $InsP_6$ED also varied widely between feeds for both calculated passage rates and was highest for faba beans, maize and DDGS ($InsP_6ED_5$: 93, 93 and 92%; $InsP_6ED_8$: 91, 90 and 89%, respectively),

Table 2 *Concentrations of phytate ($InsP_6$) and myo-inositol pentakisphosphate ($InsP_5$) in the examined single and compound feeds[1] (µmol/g DM and g/kg DM)*

		$InsP_6$		$InsP_5$	
Feed		µmol/g DM	g/kg DM	µmol/g DM	g/kg DM
Maize		10.7	7.0	0.3*	0.2*
Wheat		12.4	8.2	0.3*	0.2*
Barley		9.6	6.3	0.3*	0.2*
Faba beans		21.7	14.3	2.7	1.6
Soybeans		21.8	14.4	3.9	2.2
Soybean meal		25.8	17.0	3.8	2.2
Rapeseed meal		36.5	24.1	5.4	3.2
Sunflower meal		49.9	32.9	7.5	4.4
DDGS		7.0	4.6	3.9	2.2
CF1	Mash	13.2	8.7	2.0	1.2
	Pellet	13.5	8.9	1.5	0.9
CF2	Mash	21.8	14.4	2.9	1.7
	Pellet	19.1	12.6	2.5	1.5

DDGS = dried distillers' grains with solubles; CF1 = compound feed 1 (containing 10% maize, 46% barley, 16% faba beans, 18% soybeans, 5% soybean meal, 5% DDGS on DM basis); CF2 = compound feed 2 (containing 32% maize, 12% wheat, 16% faba beans, 8% soybean meal, 17% rapeseed meal, 10% sunflower meal, 5% DDGS on DM basis).
*Below limit of quantification, approximate value (mean between limit of detection and limit of quantification).
[1]Chemical composition of the feeds besides inositol phosphates published by Grubješić *et al.* (2019).

followed by soybeans, wheat and barley ($InsP_6ED_5$: 89, 82, 80%; $InsP_6ED_8$: 85, 76, 74%, respectively; Table 3). In the oilseed meals, $InsP_6ED$ was lowest with values for $InsP_6ED_5$ and $InsP_6ED_8$ of 76 and 66% for SBM, 75 and 65% for SFM and 59 and 48% for RSM, respectively. A significant lag time was only calculated for SBM (3.6 h) and SFM (3.1 h).

Degradation parameters and effective degradation of phytate from compound feeds

In CF, fraction *a* was significantly higher for both CF Pellets compared to their respective Mash (CF1: 71 *v.* 56%, CF2: 56 *v.* 38%; Table 4). The same was observed for $InsP_6ED_5$ (CF1: 91 *v.* 86%, CF2: 85 *v* 80%) and $InsP_6ED_8$ (CF1: 88 *v.* 81%, CF2: 80 *v.* 72%). For fraction *c*, no interactions between feed and type existed, but the degradation rate was significantly higher for CF2 compared to CF1 (17.5 *v.* 11.2%/h). Calculated values for fraction *a*, $InsP_6ED_5$ and $InsP_6ED_8$ did not differ from observed values for CF1 Mash but were lower than the observed values of CF1 Pellet. For CF2, calculated values for fraction *a*, $InsP_6ED_5$ and $InsP_6ED_8$ were lower than the observed values of CF2 Mash and CF2 Pellet.

Concentrations of lower inositol phosphates after different incubation times

Isomers of $InsP_5$ were detected in the bag residues of all incubated feeds except for maize. Concentrations of $InsP_5$ in the bag residues during the course of incubation are shown in Figure 1. Compared to the concentrations in the feeds, the $InsP_5$ concentrations in the bag residues initially increased for wheat, barley, RSM, SFM and CF2 Mash after 2 or 4 h but decreased quickly afterwards. Only traces of $InsP_5$ were detected in the bag residues after 16 h (wheat, barley, soybeans, faba beans, DDGS) or 24 h of incubation (SBM, RSM, SFM, CF1, CF2). Inositol phosphates lower than $InsP_5$ were only found in the form of $InsP_4$ in the bag residues of SFM (after 2 and 4 h) and RSM (after 4 h of incubation), but the concentrations were negligible (data not shown).

Near-infrared spectroscopy calibrations

The calibration based on data set 7 showed the highest R^2 values and the lowest error measurements (Table 5, Figure 2). For all data sets, the first derivation of the spectra showed the best performance. With the exception of data set 4, the calibration based on the wavelength segment of 1250 to 2450 nm was chosen for all data sets as the best performing one. Deviation of the prediction from the chemically determined $InsP_6$ concentration against the predicted value was homogeneously distributed across the whole range of predictions (Figure 2). The $InsP_6$ concentrations of feeds and bag residues derived from data set 7 were then used to calculate $InsP_6ED$ NIRS for comparison with $InsP_6ED$ HPIC (Table 6). Significant differences in $InsP_6ED$ values occurred for some feeds. For wheat, barley and CF1 Mash, $InsP_6ED_8$ NIRS was up to 10 percentage points higher

Table 3 *Ruminal degradation parameters and effective degradation of phytate ($InsP_6$) for single feeds (n = 3 animals)*

	Maize	Wheat	Barley	Faba beans	Soybeans	Soybean meal	Rapeseed meal	Sunflower meal	DDGS	Pooled SEM	*P*-values
a	63^{c}	45^{d}	44^{d}	74^{b}	62^{c}	27^{e}	0^{g}	15^{f}	77^{a}	0.66	<0.001
b	37^{e}	55^{d}	56^{d}	26^{f}	38^{e}	73^{c}	100^{a}	84^{b}	22^{g}	0.71	<0.001
c	24.9^{ab}	10.2^{d}	9.4^{d}	14.9^{bcd}	12.2^{cd}	20.7^{abc}	7.3^{d}	28.2^{a}	10.8^{cd}	3.48	0.005
lag	–	–	–	–	–	3.6^{a}	–	3.1^{b}	–	0.09	0.005
$InsP_6ED_5$	93^{a}	82^{c}	80^{c}	93^{a}	89^{b}	76^{d}	59^{e}	75^{d}	92^{a}	0.86	<0.001
$InsP_6ED_8$	90^{a}	76^{c}	74^{c}	91^{a}	85^{b}	66^{d}	48^{e}	65^{d}	89^{a}	1.11	<0.001

a = rapidly degradable fraction (%); *b* = potentially degradable fraction (%); *c* = degradation rate of *b* (%/h); lag = lag time (h); $InsP_6ED$ = effective degradation (%) of $InsP_6$ at a passage rate of 5 ($InsP_6ED_5$) and 8 ($InsP_6ED_8$) %/h.
DDGS = dried distillers' grains with soluble.
Different superscripts within a row indicate significant differences.

Table 4 *Ruminal degradation parameters and effective degradation of phytate ($InsP_6$) for compound feeds (CF1/2 Mash, CF1/2 Pellet and CF1/2 Calculated,* n = *3 animals)*

	CF1			CF2							*P*-values		
Type	Mash	Pellet	Calculated	Mash	Pellet	Calculated	Pooled SEM	CF1	CF2	Pooled SEM	CF × Type	CF	Type
a	56[b]	71[a]	57[b]	38[c]	56[b]	32[d]	0.95				<0.001	<0.001	<0.001
b	44[c]	29[d]	43[c]	61[b]	43[c]	68[a]	0.91				<0.001	<0.001	<0.001
c	10.5	11.1	12.0	18.0	20.1	14.4	–	11.2	17.5	1.65	0.442	0.014	0.662
lag	–	–	0.3[d]	2.5[b]	3.5[a]	1.0[c]	0.18				<0.001	<0.001	<0.001
$InsP_6ED_5$	86[b]	91[a]	87[b]	80[c]	85[b]	77[d]	0.73				0.022	<0.001	<0.001
$InsP_6ED_8$	81[b]	88[a]	82[b]	72[c]	80[b]	69[d]	0.87				0.030	<0.001	<0.001

a = rapidly degradable fraction (%); *b* = potentially degradable fraction (%); *c* = degradation rate of *b* (%/h); lag = lag time (h); $InsP_6ED$ = effective degradation (%) of $InsP_6$ at a passage rate of 5 ($InsP_6ED_5$) and 8 ($InsP_6ED_8$) %/h.
CF1 = compound feed 1 (containing 10% maize, 46% barley, 16% faba beans, 18% soybeans, 5% soybean meal, 5% dried distillers' grains with solubles (DDGS) on DM basis); CF2 = compound feed 2 (containing 32% maize, 12% wheat, 16% faba beans, 8% soybean meal, 17% rapeseed meal, 10% sunflower meal, 5% DDGS on DM basis).
CF Calculated = ruminal degradation parameters and effective degradation of $InsP_6$ calculated from single feeds.
Different superscripts within a row indicate significant differences.

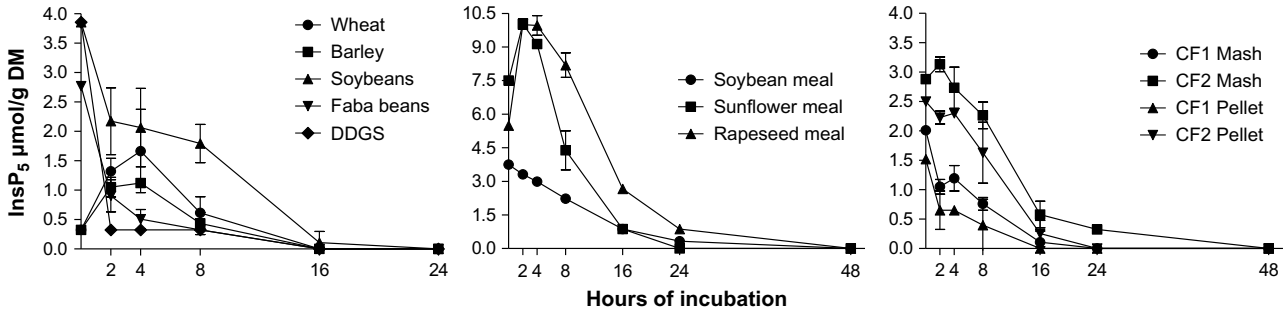

Figure 1 Concentrations of *myo*-inositol pentakisphosphate ($InsP_5$; µmol/g DM) in the bag residues of *in situ* incubated single and compound feeds at different incubation times (*n* = 3 animals; DDGS = dried distillers' grains with solubles; CF1 = compound feed 1 (containing 10% maize, 46% barley, 16% faba beans, 18% soybeans, 5% soybean meal, 5% DDGS on DM basis); CF2 = compound feed 2 (containing 32% maize, 12% wheat, 16% faba beans, 8% soybean meal, 17% rapeseed meal, 10% sunflower meal, 5% DDGS on DM basis).

Table 5 *Performance of different calibrations for estimating the phytate ($InsP_6$) concentration of single feeds, compound feeds and their bag residues after ruminal* in situ *incubation; cross-validation groups: 5*

	Settings		Calibration				Validation				
Data set	Wavelength (nm)	D,G,S	Factors	Samples Available/ used	SEC (µmol/g)	R^2	SEP (µmol/g)	R^2	Bias (µmol/g)	Slope	Intercept (µmol/g)
(1)	1250 to 2450	1,8,8	15	127/127	3.6	0.95	5.3	0.90	−0.76	1.04	−1.55
(2)	1250 to 2450	1,8,8	15	259/259	3.9	0.94	4.5	0.93	−0.43	1.02	−0.88
(3)	1250 to 2450	1,8,8	15	229/229	4.0	0.94	5.1	0.92	−0.61	1.03	−1.23
(4)	680 to 2500	1,8,8	5	95/87	1.5	0.92	4.2	0.66	<0.01	1.00	<0.01
(5)	1250 to 2450	1,8,8	15	156/156	3.2	0.97	4.6	0.95	−0.61	1.03	−1.24
(6)	1250 to 2450	1,8,8	15	220/220	3.7	0.95	4.2	0.94	−0.32	1.02	−0.64
(7)	1250 to 2450	1,8,8	15	117/117	3.3	0.97	3.9	0.97	−1.01	1.04	−2.06

D,G,S = Derivation, Gap, Smooth; R^2 = squared correlation coefficient; SEC = Standard Error of Calibration; SEP = Standard Error of Prediction; data set 1: all values for feeds and bag residues of the present study; data set 2: all values for feeds and bag residues of the present study and the additional studies (Seifried *et al.*, 2016 and 2017; Haese *et al.*, 2017c, Krieg *et al.*, 2017); data set 3: data set 2, but excluding all values for rye and triticale; data set 4: only values for feeds and bag residues from grain samples of the present study and the additional studies; data set 5: data set 2, but excluding all values for grain samples; data set 6: data set 2, but excluding all values for compound feeds; data set 7: data set 2, but excluding all values for compound feeds and grain samples.

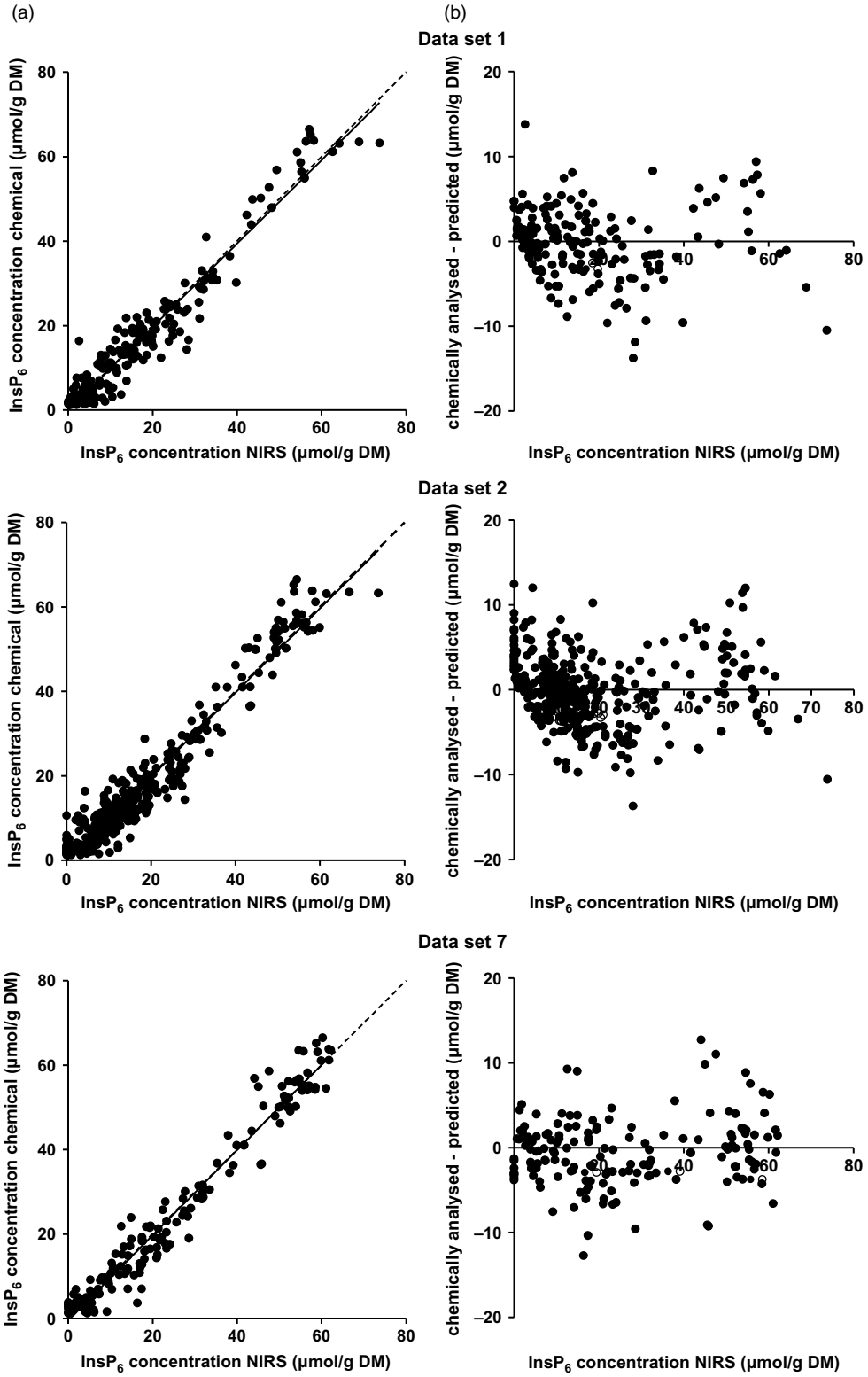

Figure 2 (a) Phytate ($InsP_6$) concentrations (predicted with near-infrared spectroscopy (NIRS) vs. chemically analysed) in samples from *in situ* studies based on data sets 1, 2 and 7, the corresponding regression line (solid line) and the bisectrix (dashed line). (b) Difference between NIRS predicted and chemically analysed $InsP_6$ concentrations in samples of *in situ* studies. Negative values were treated as zero.

compared to $InsP_6ED_8$ HPIC. On the other hand, $InsP_6ED_8$ NIRS for maize, SBM and SFM was up to 16 percentage points lower compared to $InsP_6ED_8$ HPIC. For the other feeds (faba beans, soybeans, RSM, DDGS, CF1 Pellet, CF2 Mash and CF2 Pellet), $InsP_6ED$ NIRS and $InsP_6ED$ HPIC did not differ significantly.

Table 6 *Effective degradation of phytate ($InsP_8$) at a passage rate of 5 ($InsP_6ED_5$) and 8 ($InsP_6ED_8$) %/h calculated from $InsP_6$ concentrations predicted with near-infrared spectroscopy (NIRS) or chemically (HPIC) analysed*

Feed	Maize		Wheat		Barley		Faba beans		Soybeans		Soybean meal		Rapeseed meal		Sunflower meal		DDGS		CF1 Mash		CF1 Pellet		CF2 Mash		CF2 Pellet		*P*-values		
Method	NIRS	HPIC	NIRS	HPIC	NIRS	HPIC	NIRS	HPIC	NIRS	HPIC	NIRS	HPIC	NIRS	HPIC	NIRS	HPIC	NIRS	HPIC	NIRS	HPIC	NIRS	HPIC	NIRS	HPIC	NIRS	HPIC	Pooled SEM	Feed × Method	Method	Feed
$InsP_6ED_5$	85^{fg}	92^{abc}	90^{cde}	82^{h}	85^{g}	80^{h}	94^{a}	94^{ab}	88^{ef}	89de	71^{j}	76^{i}	61^{k}	59^{k}	61^{j}	75^{i}	93^{ab}	92^{abc}	91^{abcd}	86^{fg}	92^{abc}	91^{bcde}	80^{h}	80^{h}	86^{fg}	85^{g}	1.2	<0.001	0.643	<0.001
$InsP_6ED_8$	80^{fg}	90^{abc}	86^{cde}	76^{hi}	79^{gh}	74^{i}	92^{a}	91^{ab}	84^{ef}	85de	59^{k}	66^{ij}	49^{l}	48^{l}	49^{l}	65^{j}	92^{a}	89^{abc}	89^{abc}	81^{fg}	90^{ab}	88^{bcd}	73^{i}	72^{i}	81^{fg}	80^{g}	1.0	<0.001	0.865	<0.001

DDGS = dried distillers' grains with solubles; CF1 = compound feed 1 (containing 10% maize, 46% barley, 16% faba beans, 18% soybeans, 5% soybean meal, 5% DDGS on DM basis); CF2 = compound feed 2 (containing 32% maize, 12% wheat, 16% faba beans, 8% soybean meal, 17% rapeseed meal, 10% sunflower meal, 5% DDGS on DM basis).
Different superscripts within a row indicate significant differences.

Discussion

Phytate degradation from single feeds

The wide variation in $InsP_6ED$ between the examined feeds proves the necessity to evaluate the ruminal degradation of $InsP_6$ individually for single feeds. The results showed that even when feeds are categorised in legume seeds (faba beans, soybeans), cereals (maize, wheat, barley) and oilseed meals (SFM, SBM, RSM), $InsP_6ED$ varies widely within these categories. For unprocessed feeds, the extent of ruminal $InsP_6$ degradation seems to be influenced mainly by localisation and binding of $InsP_6$ in the seeds (Haese *et al.*, 2017a and 2017b). However, the effects of genotype and harvest year on $InsP_6$ degradation of legume seeds and cereal grains have not yet been studied. As variation of ruminal CP degradation between barley (ED_8: 69% to 80%; Krieg *et al.*, 2018b) and wheat (ED_8: 72% to 80%; Seifried *et al.*, 2017) genotypes has been observed, this might also apply to ruminal $InsP_6$ degradation. In a previous study, we examined the correlation between CP and $InsP_6$ disappearance for different feeds and found high coefficients of determination for the linear regressions ($R^2 \geq 0.93$ for oilseed meals, $R^2 = 0.83$ for wheat; Haese *et al.*, 2017b). Therefore, factors influencing ruminal CP degradation might also affect ruminal $InsP_6$ degradation.

For processed feeds such as oilseed meals, processing conditions seem to have a major influence on the extent of ruminal $InsP_6$ degradation and might explain the relatively low $InsP_6ED$ of SBM and RSM compared to other studies. In the studies of Konishi *et al.* (1999) and Park *et al.* (1999), $InsP_6ED_8$ was 59% for RSM and 74% for SBM, while in the present study, $InsP_6ED_8$ was only 48% for RSM and 66% for SBM. Heat treatment seems to have a major influence on $InsP_6ED$, as additional heating of meals for 3 h at different temperatures (133°C, 143°C, 153°C) reduced $InsP_6ED_8$ for both RSM (46%, 42%, 14%) and SBM (65%, 57%, 45%; Konishi *et al.* (1999)). Steingass *et al.* (2013) and Broderick *et al.* (2016) found considerable variation of ruminal degradability of CP in RSM from different oil mills and explained these observations with different heating procedures during toasting. Because disappearance of CP and $InsP_6$ is correlated in oilseed meals (Haese *et al.*, 2017b), it is likely that ruminal $InsP_6$ degradation in RSM and SBM also depends on the production process and thus differs between meals from different processing plants. The same might apply to SFM where, to the best of the authors' knowledge, data on ruminal $InsP_6$ degradation have not yet been published.

As no accumulation of $InsP_{3\text{-}5}$ was observed for any incubated feed, it can be assumed that $InsP_6$ is completely dephosphorylated once this process has begun on an $InsP_6$ molecule. For poultry, it has been shown that, even when phytase is supplemented to the feed, $InsP_6$ is not completely dephosphorylated in the precaecal part of the digestive tract (Sommerfeld *et al.*, 2018). In ruminants, however, the *in vitro* study of Brask-Pedersen *et al.* (2011) as well as the *in situ* study of Haese *et al.* (2017b) suggested that the crucial step

in $InsP_6$ degradation is the cleavage of the first phosphate group and hydrolysis of $InsP_5$ and lower InsPs follows soon after. This is consistent with the results of the present study and can probably be assumed for all feedstuffs as a quite broad range of feeds was examined. Still little is known about phytase-producing bacteria and their specific phytases, but Nakashima *et al.* (2007) found two different phytase sequences in the rumen bacterium *Selenomonas lacticifex* and suggested that in this bacterium multiple phytate degrading enzymes are present. Furthermore, Li *et al.* (2014) found that phytase-producing microorganisms did not constantly secrete functional phytases, when rumen samples gained at different times after feeding were analysed. This indicates that in the rumen various phytases are available at any time leading to complete hydrolysis of $InsP_6$, whereas in non-ruminants, where diets are usually supplemented with only one specific phytase, lower InsPs do accumulate.

Additivity of phytate degradation of compound feeds and pelleting effect

Compound feeds are often pelleted, hence it is of practical value if $InsP_6ED$ can be calculated from that of single feeds. Calculated $InsP_6ED$ underestimated observed $InsP_6ED$ of both CF1 Pellet ($InsP_6ED_5$: 4, $InsP_6ED_8$: 6 percentage points) and CF2 Pellet ($InsP_6ED_5$: 8, $InsP_6ED_8$: 11 percentage points). This suggests that, at present, $InsP_6ED$ of CF cannot be calculated reliably with sufficient precision from values of single feeds. As the difference between calculated and observed values of $InsP_6ED$ was smaller for CF1, the precision of the calculation could depend on the single feeds used. So far, CF are mainly used to supply energy and CP, and their contribution to P supply has not yet been of major interest. However, depending on the constituent single feeds its contribution can be relevant, and gaining an estimate of the availability of this P source is an improvement towards precise calculation of diets. Thus, further research is required on this topic as we examined only two different CF in the present study.

Both CF1 Pellet and CF2 Pellet showed higher $InsP_6ED$ values compared to the respective Mash (CF1: $InsP_6ED_5$: 5, $InsP_6ED_8$: 7 percentage points; CF2: $InsP_6ED_5$: 5; $InsP_6ED_8$: 8 percentage points). This effect was also observed for effective degradation of CP in CF1 and CF2 (Grubješić *et al.*, 2019). As degradation rate *c* was not affected by pelleting, this effect can probably be ascribed to the increase of fraction *a* after pelleting (CF1: 15, CF2: 18 percentage points). A higher proportion of finer particles was measured after pelleting of CF1 and CF2 (Grubješić *et al.*, 2019), and it can be concluded that the increased $InsP_6ED$ in pelleted feeds derived from fine particles which were prone to leave the bag undegraded and thus increased fraction *a*. As mentioned before, heat treatment at high temperatures usually impairs ruminal $InsP_6$ degradation. Pelleting proceeded at a temperature of 50°C to 60 °C, and the exit temperature of the pellets was 80°C to 90 °C. Either this temperature was not sufficient to facilitate any structural changes decreasing $InsP_6$ degradation or the changes in particle size distribution covered this effect.

Prediction of phytate concentrations using near-infrared spectroscopy

The performance of the calibration based on data set 7 yielded the highest R^2 in the validation step and the lowest SEP of all calibrations. Thus, the difference between the chemically analysed and NIRS predicted $InsP_6$ concentrations were overall lower for data set 7 than for the other calibrations (Figure 2). However, the bias and intercept were higher for data set 7 calibrations than for the other sets. When regressions were calculated between the error of $InsP_6$ predictions and the predicted $InsP_6$ concentrations, slopes were not significant in any case. This implies that the error of the prediction did not depend on the $InsP_6$ concentration of the sample. This, in turn, means that the prediction of $InsP_6$ concentrations is possible with similar accuracy for feed samples and bag residues, where $InsP_6$ concentrations are distinctly lower due to ruminal incubation.

Overall, the performance of calibrations in the present study was not as good as the performance of calibrations for the prediction of CP concentrations in similar samples (Krieg *et al.*, 2018a). For most of the data sets, the wavelength segment of 1250 to 2450 nm was selected for prediction of CP and $InsP_6$ concentration. The aforementioned correlation between CP and $InsP_6$ concentration in different feeds (Haese *et al.*, 2017b) and the preference for the same wavelength segments support the theory of $InsP_6$ being indirectly predicted from CP. Since $InsP_6$ and CP concentrations are correlated but do not change directly proportional, this theory would also explain the lower performance of $InsP_6$ calibrations compared to the calibrations for predicting CP concentration.

The improvement of the performance of the calibrations by exclusion of cereal grains and CF suggests that strong matrix effects exist between cereal grain samples and protein feeds. No clear separation of spectra from cereal grain samples and their incubation residues from the other samples was visible (principal component analysis plot, data not shown, MATLAB, Fathom Toolbox; Jones (2014)). However, the decrease in the SEP and the increase in the R^2 upon exclusion of grain samples suggest that separate calibrations for cereal grains and protein-rich feeds should be further worked on. Assumedly, the matrix effects occur due to different interactions between $InsP_6$ and CP in cereal grains and protein feeds which result in differing degradation kinetics of CP and $InsP_6$. This probably leads to changes in the relations between $InsP_6$ and CP concentrations of feeds and bag residues which might affect protein-rich feeds to a different extent than cereal grains. Together with the previously assumed indirect prediction of $InsP_6$ by CP, this could lead to a less favourable performance of global calibrations. This theory is supported by the relatively homogenous distribution of the samples in the PCA plot. A separation of grain samples based on the error of the prediction could be expected based on the comparison of the $InsP_6ED$ values, but was not given for any of the calibrations (Figure 2). The comparison of $InsP_6ED$ NIRS with $InsP_6ED$ HPIC also indicates that the NIRS prediction of $InsP_6$ concentrations is not

yet sufficiently accurate. While no differences between $InsP_6$ED NIRS and $InsP_6$ED HPIC were observed for some feeds, $InsP_6$ED NIRS was considerably lower (e.g. 16 percentage points for SFM) or higher (e.g. 10 percentage points for wheat) for other feeds. This underlines the need for more data to develop suitable calibrations.

The authors are not aware of any study that reported calibrations to predict $InsP_6$ concentrations in ruminally incubated samples. However, calibrations do exist to predict $InsP_6$-P concentration in poultry feeds (Tahir *et al.*, 2012; Aureli *et al.*, 2017). Values of the present study expressed as $InsP_6$-P ranged from 0.23 to 12.12 g/kg, which is in a similar range as the values of Tahir *et al.* (2012) and Aureli *et al.* (2017). In the study of Tahir *et al.* (2012), the R^2 of the validation step ranged from 0.67 (maize) to 0.94 (wheat shorts) and the SEP from 0.09 g/kg (SBM) to 0.23 g/kg (maize, DDGS). Recalculation of the SEP in the present study to g/kg $InsP_6$-P resulted in slightly higher SEP values between 0.7 and 1.0 g/kg. Calibrations of Aureli *et al.* (2017) were based on a slightly bigger range of reference $InsP_6$-P concentrations 0.2) to 14.1 g/kg) and showed a comparable R^2 (0.94) and SEP (0.67 g/kg) than most of the calibrations of the present study. The slightly higher SEP values observed here are probably due to the more heterogeneous sample material (feeds and bag residues after different incubation times) compared to calibrations comprising only feedstuffs. Besides the establishment of local calibrations, the usage of other chemometric techniques than PLS might help to improve the accuracy of the prediction. First trials with data of the present study utilising artificial neural networks instead of PLS to predict $InsP_6$ concentrations delivered promising results and should be further investigated. Overall, the calibrations that were established in the present study demonstrate that $InsP_6$ can be predicted by NIRS in incubated samples of *in situ* studies as well as in feeds. However, the results also show that the used database needs to be expanded to achieve sufficient performance of the calibrations for the use in *in situ* studies.

The results of the present study indicate that the availability of $InsP_6$-P should be evaluated individually for feeds. However, to broaden the data base on ruminal $InsP_6$ degradation of different feeds establishing a fast and easy method for analysis of $InsP_6$ is a decisive factor. Predicting $InsP_6$ concentrations in feeds and bag residues using NIRS proved to have the potential to simplify the analytical step of $InsP_6$ in future *in situ* studies.

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Declaration of interest

The authors have no conflicts of interest to declare.

Ethics statement

The conduction of the study was in accordance with the German animal welfare regulations. Housing, diets and incubation procedure were approved by the Regierungspräsidium Stuttgart (Germany, approval code V319/14 TE).

Software and data repository resources

None of the data were deposited in an official repository.

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5. GENERAL DISCUSSION

Evaluation of additivity of ruminal degradation of nutrients and feeding values of single feeds in compound feeds was the first aim of the present thesis. The second aim was to investigate effects of pelleting on the ruminal degradation of nutrients and feeding values of compound feeds. To investigate the aims of the present thesis, *in situ* and different *in vitro* procedures were performed for determination of ruminal degradation of nutrients and feeding values of single and compound feeds. Most relevant advantages and limitations of those methods, as well as some methodological aspects that are useful for the correct interpretation of results are discussed in Chapter 5.1.

5.1. Methodological aspects

In the present thesis the intention was to utilise feed samples and feed processing conditions that closely mirror those used in practical feed production. This was achieved in cooperation with the commercial animal feed producer Raiffeisen Kraftfutterwerk (RKW) located in Kehl, Germany. Twelve common single feeds were selected: maize (dried at 70°C), wheat (non-processed), barley (non-processed), soybeans (full-fat, thermally treated 1–2 min at ≈180°C, urease activity was subsequently controlled), soybean meal, rapeseed meal, sunflower meal (with hulls), faba beans (non-processed), dried distillers' grains with solubles (**DDGS**; commercial name Protigrain, CropEnergiesAG, Mannheim, Germany), maize gluten (feed), wheat bran (not thermally treated), and sugar beet pulp.

Eight compound feeds were formulated, targeting CP concentrations of 16, 18, 20, 22, 24, 26, 28, and 30% of CP in DM. Compound feeds included five to seven single feeds, without addition of mineral supplements or amino acids. These eight compound feeds were produced in both mash and pelleted form (Figure 1). Some differences in CP and ST concentration between mash and pelleted compound feed 6 were found (Manuscript 1). Differences were observed using both the reference methods of chemical analysis of CP and ST, as well as using near-infrared spectroscopy (**NIRS**). This could not be explained, as pelleting is not known to influence CP or ST concentration.

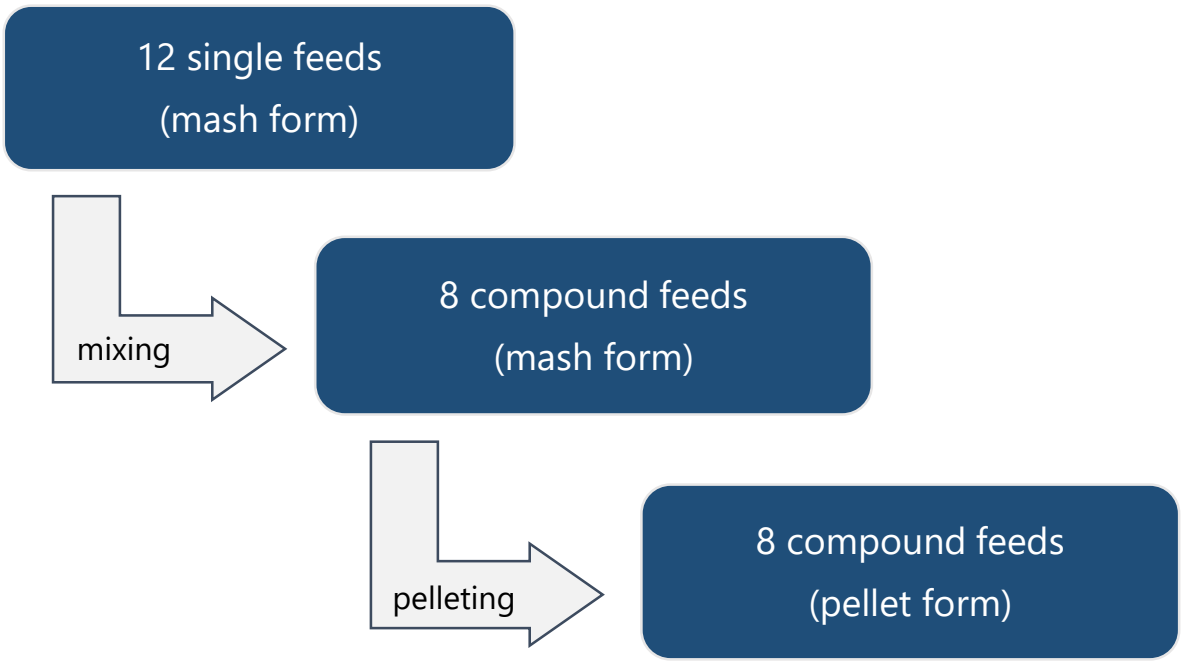

Figure 1. Overview of feed sample types used in the assays of the present thesis

In contrast to the mathematic approach of evaluation of additivity that was used in the present thesis (Manuscripts 1–3), some other more complex mathematical methods for evaluating the presence of associative effects are known, such as surface response method (Franci and Acciaioli, 1998) and mixture simplex design (Sandoval-Castro *et al.*, 2002). These methods require all single feeds to be included in same predefined steps in compound feeds in all possible combinations (Franci *et al.*, 1997). Precise pinpointing of associative effects on a specific feed is possible only when all single feeds are present in all examined compound feeds. This is practical only when evaluating a smaller number of single feeds. In the present thesis, the goal was to produce compound feeds as used on farms, formulated to target specific protein levels, including wide variety of single feeds. This implied that not all single feeds were included in all compound feeds.

5.1.1. *In situ* method

While the *in situ* method is considered a reference method for determination of RUP, it is also commonly utilised to estimate ruminal degradability of ST (Cerneau and Michalet-Doreau, 1991; Krieg *et al.*, 2017) and more recently also $InsP_6$ (Haese *et al.*, 2017), among others. The *in situ* method enables a relatively easy study of degradation of feed samples in the rumen environment (Nocek, 1988). However, there are some disadvantages of the *in situ* method.

Repeatability of *in situ* measurements was questioned in the ring study of Madsen and Hvelplund (1994). They found significant intra-laboratory differences, and concluded

they were mostly due to the sample preparation and processing, and because different bags were used for incubations. Standardisation of the *in situ* method has been proposed in multiple publications (Madsen and Hvelplund, 1994; Vanzant *et al*., 1998; Südekum, 2005). In the present work, an attempt was made to minimise possibilities for methodological variability using the same experimental procedure for all single and compound feeds, with sufficient repetitions per feed sample and using three animals fed the same ration. In the research of Vik-Mo and Lindberg (1985) on additivity of ED of DM and CP in binary feed mixtures, two different diets were fed to cows, and the significant effect of diet on EDCP was reported. In the present thesis, the cow's diet remained the same during the whole duration of the *in situ* trial, and all the feeds were treated the same way, so that ruminal degradation characteristics of single and compound feeds could be adequately compared.

Bag microenvironment differs in a multitude of ways from the ruminal conditions including lower pH, feed samples in bags having smaller volume of exchange with the rumen contents (Marinucci *et al*., 1992; Nozière and Michalet-Doreau, 1996), with bacterial population itself differing from the one outside of the bag (Meyer and Mackie, 1986), although the difference was higher for cellulolytic bacteria (that primarily digest cellulose) than for amylolytic bacteria (that digest CP and ST). Thus, for mixtures of single concentrate feeds the bacterial population should be similar within and outside of the bags. The ruminal microbial ecosystem is affected by changes in the diet (Nocek, 1988). Similarly, the bag ecosystem may presumably be affected by the feed sample. Goelema *et al*. (1998) considered that results of Vik-Mo and Lindberg (1985) and Chapoutot *et al*. (1990) indicate that incubation of single feeds with different protein and carbohydrate degradability improves the bag microbial ecosystem, and thus facilitates more complete degradation of feed mixtures when compared with single feeds. Since different single feeds would have a specific influence on the bag ecosystem, this would also be true for different compound feeds. Such change could presumably affect the degradation of single feeds when incubated together, and would be detected as an associative effect.

Some feeds may have specific physical characteristics that may affect the passage of feed particles through the bag pores. This was previously found for maize gluten meal (Stern *et al*., 1983), because of its viscosity that may lead to feed sample inside of the bag to stick when wet. This could manifest as an associative effect in compound feeds where maize gluten meal is included. In the present work, maize gluten feed was used, but the effect of its presence on the nutrient disappearance from bags could not be tested, due to only two inclusion levels of maize gluten in compound feeds (Manuscript 1).

Ruminal microorganisms attach more easily to damaged cell wall surfaces, which is promoted with mastication (Lathan, 1980). However, the *in situ* method implies incubation of bags with feed samples in the rumen, skipping the previous part of the digestive tract. Therefore, all feed samples were milled through the same 2 mm screen. The effect mastication may have on the increase of the moisture of feed samples and to increased microbial attachment was offset in the present work with soaking of bags in water before incubation.

All feed samples in the present thesis were ground through the same screen for each experiment. For the *in situ* procedures, the recommended 2 mm screen was used (Vanzant *et al*., 1998). However, samples were also pre-ground in the feed mill, using a 3 mm screen. To get a good description of particle sizes, particle size distributions were determined using wet sieving (Manuscript 1). Wet sieving method was chosen instead of dry sieving. Kennedy (1984) advised for using wet sieving for trials involving gastro-intestinal tract measurements. During the wet sieving procedure, clogging of the smallest of the seven sieves (diameter of 0.063 mm) in form of a layer of white foam was noticed. An attempt was made to prevent this by increasing the pre-soaking of samples from 1 to 24 h, and using the enzyme amylase to facilitate ST degradation, but neither of these attempts solved the persisting issue of clogging. Finally, the smallest sieve had to be removed for all pelleted compound feeds. The results of particle size distributions indicated that pelleting increased the share of smaller particles across all compound feeds, which is consistent with previous studies (Engberg *et al.,* 2002; Amerah *et al.,* 2007; Abdollahi *et al.,* 2011). This influenced the results of *in situ* study by increasing the passage of small feed particles through the bag pores, as described below.

An attempt was made to model the particle size distributions and to calculate the average particle size, as described in Siegert *et al*. (2018). The resulting regression inflection points ($\mathbf{y_1}$) can be interpreted as average particle size provided that determined y_1 are higher than the smallest sieve size because particles smaller than the smallest sieve size are not considered in regression calculations. For wheat, barley, soybeans, faba beans, mash compound feed 4, and all pelleted compound feeds the estimated y_1 was smaller than the smallest sieve size (0.063 mm for single feeds and mash compound feeds, 0.125 mm for pelleted compound feeds) and negative in some cases. Therefore, y_1 could not be interpreted as average particle size for those feeds. Accordingly, only size distributions were presented in Manuscript 1 for all feed samples. Since other methods of particle size measurement are either costly (laser diffraction) or time-consuming (microscopy), some novel methods of prevention of clogging during wet sieving should be explored. This may be hard to accomplish however, as chemical methods would probably affect different single feeds in the mixture

differently, and would affect the evaluation of additivity. The increase of sieve sizes would be in the present study impractical, because the sieve size less than a diameter of *in situ* bag pore size (0.075 mm) was necessary for adequate interpretation of data.

Results of *in situ* studies heavily depend on the particle size of feed samples (D'Mello, 2000). Michalet-Doreau and Cerneau (1991) measured particle sizes of concentrate feeds ground through the same screen. They noticed differences in the average particle size among feeds, which also affected N loss from bags. Feed particle size may affect microbial access to the substrate (Nozière and Michaelet-Doreau, 2000). Gerson *et al*. (1988) found that microbial colonisation of feed particles is inversely related to its particle size, however this was not found for feed particles within *in situ* bags (D'Mello, 2000). Therefore, even if pelleted compound feeds had a higher share of fine particles than mash, the influence of microbial colonisation probably had no influence on results of *in situ* nutrient disappearance in the present study.

However, the disappearance of nutrients from bags may be overestimated if small particles leave the bags undegraded. The feed particles lost through bag pores during the *in situ* incubation differ in their solubility in the rumen (Prestløkken, 1999). If the rate and the extent of degradation of particles lost from bags is equal to the degradation of particles within bags, the correction for particle loss can be applied. Weisbjerg *et al*. (1990) suggested a CP degradation correction for the small particle loss. This correction was tried but eventually not applied, as in some samples the water-soluble N appeared higher than the washout fraction (Manuscript 1). Those samples were soybean meal, rapeseed meal, sunflower meal, faba beans, sugar beet pulp, and mash compound feeds 6, 7, and 8.

The bag pore size was shown to significantly influence ED estimates, but the choice of the correct pore size is dependent on the feed (Seifried *et al*., 2015). Bag content degradation is limited by pore size of bags, with the minimum pore size being 10 µm and the upper limit depends on the feed particle size (Lindberg *et al*., 1984). If the pore size is too small, incubated feed will not come in sufficient contact with ruminal microbiota, and the ED will be underestimated. If the pore size is too big, some of the disappearance from the bag will be not due to microbial degradation, but particle loss through the bag, and the ED will be overestimated (López, 2005). The most commonly used recommended pore sizes lie within the range of 40–60 µm (Vanzant *et al*., 1998), and the pore size of 50 µm was chosen in the present thesis. However, the choice of 50 µm may have an influence on ST degradation (Seifried *et al*., 2015) due to so-called secondary ST loss. This influence could be uneven among single feeds used in the present study, and therefore among compound feeds. However, $EDST_{IN\_SITU}$ values were

relatively high in all compound feeds, which makes additivity more likely to be found (Manuscript 1).

Another critical consideration is sample size to bag surface area ratio (Hristov *et al.*, 2019), which was equal for both single and compound feeds in the present thesis, to ensure adequate comparison of calculated and observed values. This ratio was in the present work 20 mg/cm^2, which was at the upper border of the most commonly recommended range of values (10 –20 mg/cm^2; Vanzant *et al.*, 1998).

During post-incubation washing, feed residues inside the bags can still retain significant microbial mass (D'Mello, 2000) and can affect calculated rumen degradability (Rodríguez and González, 2006), although the effect in concentrate feeds is smaller than in forages. Careful rinsing of bags in warm water and washing them in a commercial washing machine after the incubation was performed to address this, and it was presumed that microbial remains did not affect the results of the *in situ* study for samples used in the present thesis.

Following *in situ* incubation and chemical analyses of residues, the disappearance of DM, CP, ST, and $InsP_6$ was used to compute ED_{IN_SITU} values. Based on bag losses, parameters *a, b, c,* and lag of degradation were calculated. There are multiple ways to calculate a_{IN_SITU} from the 0-h incubation disappearance, yet the choice of calculation is rarely reported in scientific studies. The a_{IN_SITU} can either be *calculated* as a mean of loss from 0-hour incubation from three (or more) bags; or *estimated* for each animal separately using regression or as an estimated mean value for 0-hour incubation loss. The former is dependent on the feedstuff, while the estimations for each animal are dependent on the animal. In the present work, the choice was made to calculate a_{IN_SITU} as an arithmetic mean of loss from 0-hour incubated bags. This means that a_{IN_SITU} values were repeated measurements, and not statistical replicates, and that *a* values could be compared only numerically. This was done for ruminal DM, CP, and ST degradation. However, compared to the literature data, ruminal $InsP_6$ degradation of rapeseed meal was higher. This may have been caused by double milling of the feed sample (both in the feed mill and for the *in situ* procedure), resulting in large washout of feed particles from the bag. To alleviate a possible bias of the estimations, a_{IN_SITU} was estimated for each animal separately for ruminal $InsP_6$ degradation of all single and compound feeds.

Two main mathematical models are commonly used to plot the degradation curve, one with the lag phase and one without. The lag phase represents the time between the start of the incubation and start of disappearance from bags (Figure 2). This is usually related to the feed examined, with some feeds showing a degradation with lag more commonly than others. The lag of degradation was also observed for some samples of

single feeds in the present thesis. Lag of CP degradation was present in one or more animals for maize, wheat, soybean, soybean meal, rapeseed meal, sunflower meal, and faba beans (Manuscript 1), for ST degradation in faba beans (Manuscript 1), and for $InsP_6$ degradation in soybean meal and sunflower meal (Manuscript 3). Due to the main goal of the present thesis being calculation of values of compound feeds from single feeds, the same mathematical model had to be used for all single and compound feeds. It was presumed that when lag equals zero, the formula will equate to that without lag.

The ruminal degradation of DM, CP, ST, and $InsP_6$ was calculated according to Ørskov and McDonald (1979) without inclusion of the lag phase:

$$Y = a + b \cdot [1 - e^{-c \cdot t}]$$

and with the inclusion of the lag phase according to McDonald (1981) modified by Südekum (2005):

$$Y = a + b \cdot [1 - e^{-c \cdot (t \cdot lag)}]$$

Therein, *Y* is degradation after *t* hours, *a* (%) is rapidly degradable fraction, *b* (%) is potentially degradable fraction, *c* (%/h) is rate of degradation of *b,* and lag (h) is lag time of degradation (Figure 2).

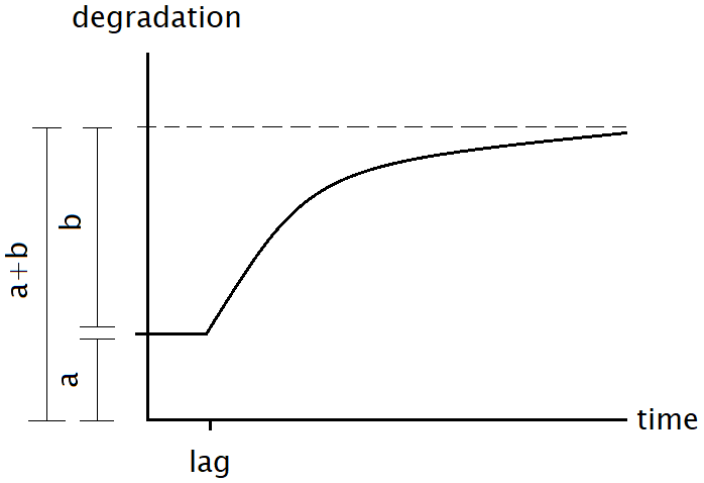

Figure 2. Schematic principle of the *in situ* degradation kinetics of nutrients in the rumen (McDonald, 1981; modified by Südekum, 2005). *a* = rapidly degradable fraction (%); *b* = potentially degradable fraction (%); *a*+*b* = maximal degradable fraction (%); lag = lag time (h).

The ED values of DM, CP, and ST were calculated after McDonald (1981) modified by Südekum (2005) (Manuscript 1) for *k* of 5%/h and 8%/h chosen to represent levels of feeding of low yielding dairy cows or beef cattle and high producing dairy cows,

respectively (AFRC, 1984). Therein, *a*+*b* (%) is the maximal degradable fraction. The calculation of ED of $InsP_6$ was described in Manuscript 3, including equations with and without lag, depending on the feed sample.

Prestløkken (1999) suggested that using the model with lag phase could probably increase the correspondence between calculated and observed values in trials evaluating additivity of ED_{IN_SITU} values. However, no differences were found between the two models for $EDCP_{IN\_SITU}$ and $EDST_{IN\_SITU}$ values in the present work. Comparison of $EDCP_{IN\_SITU}$ and $EDST_{IN\_SITU}$ values estimated using models with and without lag differences showed no or only negligible differences (Annex 1) and the model with lag phase was chosen for all feed samples and for CP and ST. In Manuscript 3, the major aim was to characterise ruminal $InsP_6$ degradation of single feeds. For this, it was important to use the models with or without lag phase according to each single feed. Because only two compound feeds were tested, the sample size was too low to compare the additivity of $InsP_6$ degradation of single feeds in compound feeds using models with and without lag phase.

Pelleted compound feeds could have been incubated *in situ* intact or milled. When submerged in water, all pellets in the present thesis readily dissolved in a matter of minutes, which was presumed to hold true in the rumen due to contact with rumen fluid. The choice was made in favour of milling for methodological consistency among all samples.

5.1.2. *In vitro* methods

Gas production methods are acknowledged as suitable for evaluation of associative effects among feeds (D'Mello, 2000). To estimate ME values from GP and proximate nutrients, different equations specific for feed types are used. In the present thesis, the ME values were initially calculated according to the feed groups (Manuscript 2): for wheat, barley, faba beans, maize gluten, and wheat bran according to Krieg *et al.* (2017); soybeans, soybean meal, rapeseed meal, sunflower meal, DDGS, and sugar beet pulp according to Menke and Steingass (1988); and compound feeds according to GfE (2009). However, this makes evaluation of ME additivity difficult, since different equations are used for single and compound feeds. Therefore, it was not surprising that calculated and observed ME values strongly differed (Manuscript 2). Alternatively, calculation of ME using equations that were developed for a wide range of feeds and mixtures of feeds was attempted. The results of this approach are discussed in Chapter 5.2.2.

In the eHGT method, uCP values are estimated (Manuscript 2). In an additional step, uCP can be separated into RUP and MCP (Steingaß and Südekum, 2013). This could enable comparison of *in vitro* RUP values to the values determined *in situ* (Manuscript 1). However, this was not possible for compound feeds in the present thesis since not all feeds satisfied the requirement of having more than 20 g of CP per MJ of ME (Steingaß and Südekum, 2013). This was the case for maize, wheat, barley, wheat bran, and sugar beet pulp, and compound feeds 1, 2, 3, 4, 5, and 6.

Animal-based ID_{RUP} is commonly determined by using the mobile nylon bag technique, but this requires ruminally and duodenally fistulated cows (NRC, 2001; Calsamiglia *et al*., 2010). Because of this, the non-invasive *in vitro* method for estimating ID_{RUP} was developed by Calsamiglia and Stern (1995). While enzymatic methods can be useful for comparing the ranking of feeds (Nocek, 1988), they may not be precise enough for accurate estimation of ID_{RUP} for each single feed necessary for evaluation of additivity (Manuscript 2). No previous research that has applied this method for the evaluation of additivity could be found.

5.1.3. Near-infrared spectroscopy

Near-infrared spectroscopy is a type of vibrational spectroscopy that utilises the near infrared region of the electromagnetic spectrum (from about 700 to 2500 nm) for quantification of interaction of near-infrared electromagnetic waves with the sample (Pasquini, 2003). Different absorption bands can be detected using NIRS, especially of organic bonds. The NIRS can detect C-C, C-H, and O-H bonds that are present both in CP and ST (Burns and Ciurczak, 2008). Additionally, the N-H bonds are related to CP, but not to ST (Krieg, 2017), allowing for differentiation. Using detected absorption bands, concentration of CP and ST in feed samples can be predicted. Therefore, NIRS technique can be used as an inexpensive alternative to chemical analysis for the routine estimation of CP and ST (Corson *et al*., 1999). A more detailed description of the methodology is given in Manuscripts 1 and 3. It is also a non-destructive method of analysis which makes it especially useful for analysis of samples like *in situ* residues, where the amount of sample material for analysis is small. Concentrations of N and ST in grains and *in situ* residues were predicted accurately using NIRS for samples of maize, wheat, barley, rye, triticale, and peas (Krieg *et al*., 2018).

In the present thesis the calibrations for single and compound feeds as well as for *in situ* residues of all single and compound feeds were developed for N and ST (Manuscript 1) and $InsP_6$ (Manuscript 3). Existing calibrations from the Institute of Animal Science (University of Hohenheim) were expanded with samples from the present work to be able to use NIRS as alternative to chemical analysis in the additivity

trials. The calibration performance was evaluated by an independent validation dataset. Feed samples were split into calibration and validation sets based on origin and concentration of N and starch. Predicted N and ST values corresponded well to the reference analysis. Therefore, prediction of N and ST was deemed suitable for feed samples and *in situ* bag residues of single and compound feeds. Separate calibration of protein rich single feeds (rapeseed meal, soybeans and soybean meal) was suggested for further NIRS calibrations concerning N, because Mahalanobis distance indicated that these samples differed from others in the set.

Besides N and ST, NIRS was used to predict the $InsP_6$ concentration in two selected compound feeds and single feeds contained therein (Manuscript 3). Molecules containing strong bonds between atoms (usually C, N, O, bond with H) absorb frequencies specific for those bonds (Manley and Baeten, 2018). Potential absorption bands relevant for $InsP_6$ are O-H bonds, but hypothetically also strong bonds between C, O, and P. This may have an implication on detection of lower InsP isomers, due to small differences from $InsP_6$ on a molecular basis. Degradation of CP and ST changes their molecular structure, however lower InsP isomers have small differences from $InsP_6$ on a molecular basis, and may be detected as $InsP_6$. Prediction of $InsP_6$ concentrations using NIRS was previously successful in a variety of feed samples (De Boever *et al.*, 1994; Olnood *et al.*, 2011). However, overall precision of $InsP_6$ prediction in the present thesis was considered to be small. The performance of calibrations was affected by inclusion of different feed types: prediction of $InsP_6$ concentration was more accurate in samples and *in situ* residues of legumes when compared to cereal grains. This may be related to the phytate-protein complexes that exist in legumes, but not in cereal grains, as discussed in Chapter 5.5. Thus, it was suggested that calibrations for predicting $InsP_6$ are kept separate for samples of cereal grains and protein rich feeds in future studies.

The $InsP_6$ concentrations predicted with NIRS were used to compute ED_{InsP6} values for single and compound feeds, which were compared to ED_{InsP6} values computed from $InsP_6$ concentrations determined with the reference chemical analysis. This was possible with high accuracy for samples of soybeans, rapeseed meal, faba beans, DDGS, compound feed 4 in pelleted form, and compound feed 5 in both mash and pelleted form. However, significant differences between two approaches were seen in maize, wheat, barley, soybean meal, sunflower meal, and compound feed 4 in mash form. Accuracy of predictions probably suffered from a relatively small number of samples, leading to high errors of prediction. Therefore, separate calibrations for those single feeds and more samples are necessary to achieve satisfactory prediction of ED_{InsP6} values.

5.2. Additivity of ruminal degradation of nutrients and feeding values of single feeds in mash compound feeds

The additivity of feeds in feed mixtures was evaluated in the past, and different positive and negative associative effects among feeds have been found. The majority of additivity research has been concerned with forage-concentrate feed interactions in regards to fibre digestibility or energy concentration. Positive associative effects commonly occur when the deficit of a nutrient (such as protein) in forage is alleviated with the addition of grains. Negative associative effects are known to occur in dairy cows mostly at high feed intake (Huhtanen, 1991), for example when the higher share of concentrate feeds rich in easily degradable carbohydrates inhibits the digestibility of forages. This occurs when cellulolytic bacteria struggle to adapt to lowered ruminal pH levels caused by rapid degradation of easily degradable carbohydrates to volatile fatty acids and lactic acid (Russell and Wilson, 1996). Higher feed intake level is also related to shorter retention of digesta in the rumen, which together with the change of the rumen environment as a result of pH change can lead to overestimation of energy intake (Huhtanen, 1991). In diets fed at or below the maintenance level, negative associative effects were previously not observed, but in highly productive animals they can be large (Mould, 1988). Concentrate feeds are the main source of CP and energy in TMR for high-yielding dairy cows. Chapoutot *et al*. (1990) found that the observed ruminal DM degradation was higher than calculated in mixtures of maize, barley, lupins, and maize gluten. Still, the knowledge of possible associative effects among concentrate feeds is scarce, particularly regarding CP and ST degradation. Causes of associative effects that occur related to forage are not anticipated in concentrate feed mixtures. In contrast to fibrous feeds, the possible change of ruminal pH has a small influence on amylolytic bacteria that carry out the degradation of CP and ST, and the lack of nutrients such as protein and energy in concentrate feeds is not likely to occur. Still, some previously unknown interactions among concentrate feeds may exist. In the following sections, additivity of single feeds in mash compound feeds is discussed first regarding protein degradation, and subsequently regarding energy and related values. An overview of absolute and relative differences between calculated and observed values of compound feeds is given in Annex 2.

5.2.1. Additivity of protein values of single feeds in mash compound feeds

Dietary CP varies widely among feeds in its concentration, as well as ruminal degradability, intestinal digestibility, and amino acid composition of RUP (NRC, 2001). Dietary CP can be separated based on its degradability in the rumen into RUP and RDP. According to NRC (2001), the concentrations of RUP and RDP in feed CP are discussed

as non-static values, being closely related to ruminal passage rate. To evaluate feeding values of feeds, knowledge on approximate share of both RUP and RDP is crucial. In the present thesis, ruminal CP degradability of single and compound feeds was evaluated using the *in situ* method. Additivity of ruminal CP degradation was previously proven in binary mixtures of soybean meal, fish meal, barley, and grass silage (Vik-Mo and Lindberg, 1985), and barley, canola meal, maize gluten meal, and barley silage (Murphy and Kennelly, 1987). Prestløkken (1999) found that observed $EDCP_{IN\_SITU}$ values in cereal and protein mixtures were smaller than calculated by up to 5 percentage points (**pp**). However, deeper insight into additivity of CP degradation of a bigger choice of different single concentrate feeds was deemed necessary.

In the present thesis, **effective degradation of CP in the rumen** was considered as additive (Manuscript 1). The differences between calculated and observed $EDCP_{IN\_SITU}$ values were not higher than 5 pp for passage rate of 8%/h (Figure 3). These differences were not related to the CP concentration of compound feeds.

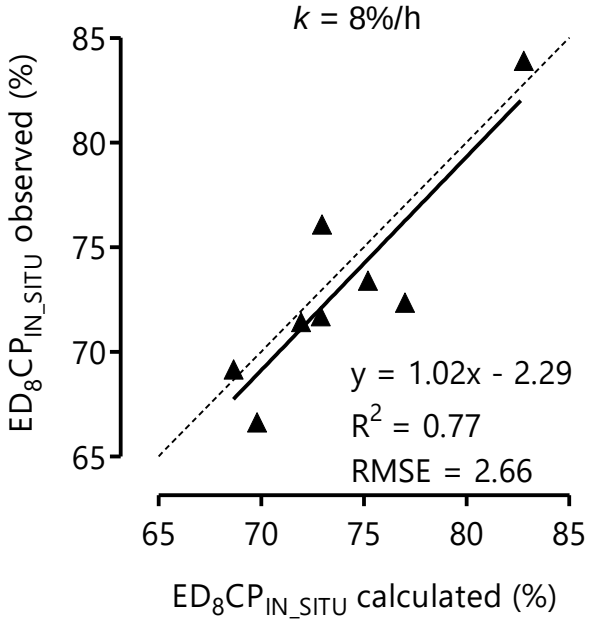

Figure 3. Comparison of calculated and observed $ED_8CP_{IN\_SITU}$ values of compound feeds in mash form

While additivity was given for $ED_8CP_{IN\_SITU}$, the observed cCP_{IN_SITU} (Figure 4) and lag of CP degradation (Manuscript 1) were significantly smaller than calculated. The observed aCP_{IN_SITU} values were in most compound feeds smaller than calculated. The bCP_{IN_SITU} differed negligibly (Manuscript 1). This indicates that a smaller amount of CP left the bag at the 0-h incubation time point than calculated in most compound feed samples (Table 1), but the remaining CP in the bag degraded with a smaller delay and with higher rate of degradation than calculated.

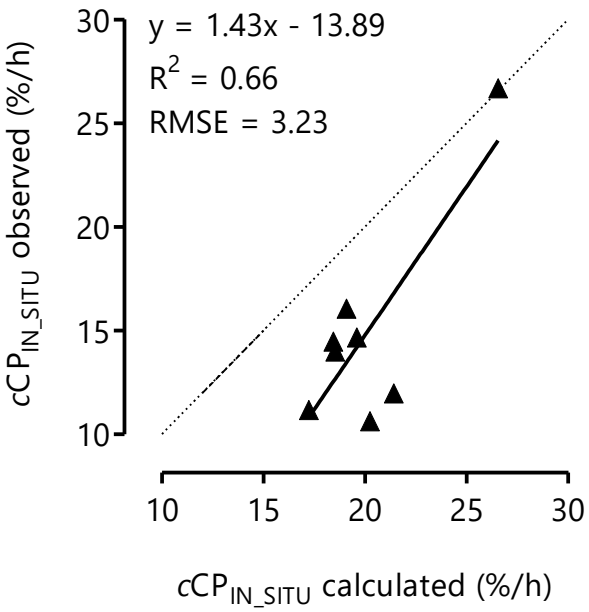

Figure 4. Comparison of calculated and observed cCP$_{IN_SITU}$ values of compound feeds in mash form

Therefore, even though individual degradation parameters were not always additive, the additivity of EDCP$_{IN_SITU}$ was given. Current feeding systems utilise RUP or uCP values of feeds for estimation of protein feed values and animal protein requirements. Future feeding systems may also use individual ruminal degradation parameters to more accurately predict feeding value of the complete diet, especially regarding synchronous ruminal degradation of CP and ST leading to better utilisation of those feed nutrients for ruminal microbial growth. Furthermore, individual degradation parameters can be used to calculate ED values for any given k, which can be more useful than publishing only ED values for fixed k values (usually 2, 5, 6, or 8%/h). For these reasons, non-additivity of cCP$_{IN_SITU}$ may become an issue for practical feed formulation in the future.

Differences between calculated and observed disappearance of CP at individual incubation time points in compound feeds existed only up to 16 h of incubation, with a maximum overestimation of 8 and 7 pp in compound feeds 4 and 5, respectively (Table 1). This difference in calculated and observed CP disappearance was also reflected in significantly smaller observed ED$_8$CP$_{IN_SITU}$ than calculated. In most compound feeds, disappearance of CP in early hours of incubation was overestimated, but in compound feeds 1 and 2 CP disappearance was underestimated, especially in the latter (up to 8 pp at 2 h of incubation), and this also caused the significantly higher observed ED$_8$CP$_{IN_SITU}$ than calculated. Differences between calculated and observed disappearance of CP after 16 h of incubation were miniscule in all compound feeds.

Table 1. Comparison of calculated and observed crude protein disappearance (%) from *in situ* bags per incubation time point (h) in mash compound feeds

Compound feed		Incubation time point								
		0	2	4	6	8	16	24	48	72[1]
1	calculated	34	39	52	61	67	87	96	98	99
	observed	35	43	55	62	66	87	96	98	98
	difference	*1*	*4*	*4*	*1*	*-1*	*-1*	*0*	*0*	*0*
2	calculated	39	50	60	68	75	88	92	96	97
	observed	40	58	67	72	81	88	92	95	95
	difference	*1*	*8*	*7*	*3*	*6*	*-1*	*0*	*-1*	*-1*
3	calculated	47	68	76	81	87	93	95	97	98
	observed	45	66	76	85	92	94	96	97	97
	difference	*-2*	*-2*	*0*	*3*	*4*	*1*	*0*	*0*	*-2*
4	calculated	43	59	66	70	78	91	97	98	100
	observed	37	55	62	67	72	84	95	98	99
	difference	*-7*	*-5*	*-4*	*-3*	*-6*	*-8*	*-2*	*0*	*-1*
5	calculated	32	43	54	63	71	88	94	97	98
	observed	30	42	51	62	66	81	94	96	96
	difference	*-2*	*-1*	*-3*	*0*	*-5*	*-7*	*0*	*-1*	*-2*
6	calculated	40	55	62	69	77	92	96	97	99
	observed	37	50	61	67	78	87	96	97	97
	difference	*-3*	*-4*	*-1*	*-2*	*1*	*-5*	*0*	*-1*	*-2*
7	calculated	36	47	55	65	73	91	96	97	98
	observed	32	45	57	68	71	91	95	96	97
	difference	*-4*	*-3*	*2*	*3*	*-3*	*0*	*-1*	*-1*	*-1*
8	calculated	36	50	57	65	74	91	97	99	100
	observed	30	44	55	67	73	94	93	98	99
	difference	*-6*	*-6*	*-2*	*3*	*-1*	*3*	*-4*	*0*	*-1*

[1]Not all single feeds were incubated for 72 h. Disappearance of crude protein at 72 h was assumed to be 100% for those single feeds.

The result of degradation of feed protein in rumen are peptides, amino acids, and ammonia. While the excess ammonia can be absorbed through the rumen wall and into the blood stream, all three are primarily utilised for production of MCP (NRC, 2001). The MCP has an exceptionally high nutritive value, as its amino acid profile closely mirrors that of milk and muscles (Nocek and Russell, 1988). In addition to MCP, the supply of high quality RUP is necessary for adequate continuous milk production in high-yielding dairy cows (NRC, 2001). The RUP is transported together with MCP and endogenous CP further to the small intestine. **The uCP at the duodenum** consists of RUP and MCP, and estimation of uCP using the eHGT is considered to be more precise that the estimation of RUP and MCP separately (Edmunds *et al.*, 2012). Since the additivity of CP degradation was given during rumen incubation, the additivity of uCP could also be presumed. This was shown in Manuscript 2, where differences between

calculated and observed uCP concentration for *k* = 8%/h was less than 13 g/kg DM. Such negligible differences, together with regression line slope that was close to 1 and the high R^2 value (R^2 = 0.96; Figure 5) indicated that the uCP concentration could be considered additive. Contrary to results in Manuscript 2, Zhao *et al.* (2005) found high associative effects among single feeds on estimated uCP of compound feeds. In their work, the method of uCP estimation was based on Zhao and Lebzien (2000). Some differences between the method of Zhao and Lebzien (2000) and the eHGT method (Steingaß and Südekum, 2013) included the buffer composition, sample incubation times (only 24 h vs. 8 and 24 h). Presumably the main cause of difference in results lies in mixing of liquid and solid phase within glass syringes. Zhao *et al.* (2005) used the hand shaking of samples, and reported an incomplete incubation of some feed samples. The mixing of liquid and solid phase within glass syringes in the present thesis was done using a rotary incubator (Manuscript 2), which prevented such errors.

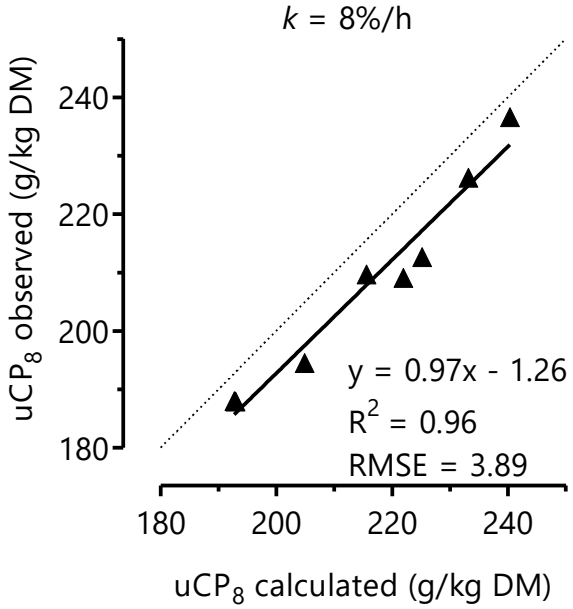

Figure 5. Comparison of calculated and observed uCP_8 values of compound feeds in mash form

As mentioned previously, the supply of CP to the small intestine consists mostly of RUP and MCP, and in small part of endogenous CP. While the MCP is known to be of high nutritional quality, the nutritional quality of RUP varies very widely among feeds (Stern *et al.*, 1985). This was determined in the Manuscript 2, where $\mathbf{ID_{RUP}}$ values varied between 18 and 83% in single feeds and between 49 and 71% in compound feeds. The three-step enzymatic method for estimation of ID_{RUP} was used in the past for a wide variety of feed samples (Calsamiglia and Stern, 1995; Woods *et al.*, 2003). However, no

publications were found on estimation of ID_{RUP} for compound feeds, or the additivity of ID_{RUP} values.

In the present work, the ID_{RUP} of compound feeds could not be accurately calculated from single feeds. Big numerical differences between calculated and observed ID_{RUP} values were determined, up to 11 pp, even though the regression line slope of 0.94 and R^2 value of 0.64 (Figure 6) indicated good fit. One reason for this can be methodological, due to the three-step method for determination of ID_{RUP} not being validated for all single and compound feeds. Therefore, some values of ID_{RUP} of single feeds may be misleading, and appearing as associative effects when summing up values to unity. Nocek (1988) discussed that enzymatic methods for estimation of digestibility are better for comparisons of digestibility among different feeds than obtaining absolute digestibility values. Lack of accurate absolute measurement of ID_{RUP} may lead to diminished suitability of using such methods for evaluation of additivity, since associative effects may appear as a sum of measurement errors.

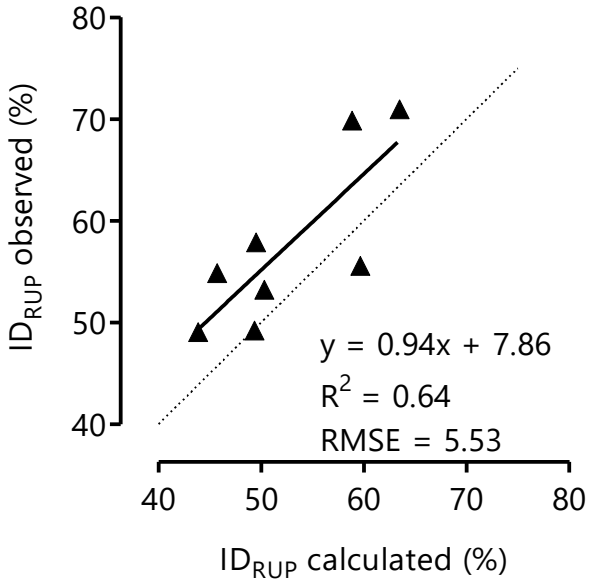

Figure 6. Comparison of calculated and observed ID_{RUP} values of compound feeds in mash form

Differences between calculated and observed ID_{RUP} may also be a result of associative effects. In that case, associative effects presumably occurred during the *in vitro* enzymatic part of the method, as the $EDCP_{IN\_SITU}$ was shown to be additive (Manuscript 1). However, when determining $EDCP_{IN\_SITU}$, multiple incubation time points (up to 72 h) are used. The associative effects found for cCP_{IN_SITU} (Figure 4) had no influence on the $EDCP_{IN\_SITU}$, but may have a bigger influence in the three-step method, where

only one incubation point (16 h) is performed. Comparison of calculated and observed CP disappearance at 16 h is given in Table 2. Observed values of CP disappearance were lower in all compound feeds when compared to calculated values, and this difference was uneven among compound feeds (from 1 to 7 pp). If solubility of CP that disappeared from bags is high, it is possible that the *in situ* procedure was a major source of associative effects among single feeds during the three-step procedure.

Table 2. Comparison of calculated and observed crude protein disappearance from *in situ* bags at 16 h of incubation in mash compound feeds

Compound feed	Calculated (%)	Observed (%)	Difference (pp)[1]
1	90	85	*-5*
2	89	85	*-4*
3	95	91	*-4*
4	95	88	*-7*
5	91	87	*-4*
6	94	92	*-2*
7	95	92	*-3*
8	97	96	*-1*

[1]percentage points

The mobile bag technique was traditionally more commonly used for estimation of ID_{RUP}. While the mobile bag method is labour and cost-intensive, validation of results from the present thesis using this method is suggested.

Chemical fractionation of CP using **CNCPS** method is quick and inexpensive compared to animal trials. In the CNCPS model, the CP can be separated into three fractions differing in their degradability: NPN (fraction A), true protein (fraction B divided into three sub-fractions (B1, B2, B3) with different rates of ruminal degradation), and unavailable protein - acid detergent insoluble nitrogen (fraction C). The CNCPS method was previously used to characterise CP in samples of various single and compound feeds (Westreicher-Kristen *et al*., 2012; Chrenková *et al*., 2014; Seifried *et al*., 2016). The CP fractions can also be used for prediction of $EDCP_{IN\_SITU}$, which is discussed in Chapter 5.6. To the authors knowledge, the additivity of CP fractions was previously not evaluated. In the present work, chemical fractionation of CP using the CNCPS method was performed for all feed samples. Additivity of CP fractions was considered to be given (Manuscript 2). Observed values of A, B1, B2, B3, and C fractions differed from calculated values non-systematically, but the numerical differences were small. Relative differences were greatest for fractions A and C. In most compound feeds the observed A fraction was higher than calculated, and observed C fraction was smaller than calculated, but the extent of associative effects was uneven among compound feeds.

Additional research on additivity of CP fractions on a wide range of single and compound feeds may be needed.

The overview of calculated and observed values of protein degradation is given in Annex 2a. The RMSE values of simple linear regressions between calculated and observed values are presented relatively to the mean observed value of each examined degradation characteristic. This enabled the possibility of relative comparison of associative effects for feeding values and ruminal degradation characteristics. The highest RMSE values relative to the mean observed values were found for *in situ* data in $lagCP_{IN\_SITU}$ at 54% and cCP_{IN_SITU} at 22%, and for *in vitro* data for CP fraction C at 38%, and fractions A and B at 18%, and ID_{RUP} at 10%. This reinforced the previous conclusions on non-additivity of individual *in situ* degradation parameters, as well as ID_{RUP}. It was discussed previously that the numerical differences between calculated and observed values of CP fractions were minor. Thus, results of regressions were not considered as the indication of non-additivity of CP fractions.

In this chapter it was shown that the additivity of EDCP and uCP values, as well as the additivity of CP fractions of single concentrate feeds in compound feeds was given. However, additivity of ID_{RUP} values was not given. For more accurate evaluation of additivity of ID_{RUP} in compound feeds, additional research using the mobile bag technique is recommended.

5.2.2. Additivity of energy values and related values of single feeds in mash compound feeds

The continuous supply of energy from concentrate feeds is crucial for high producing dairy cows, but an oversupply of easily degradable carbohydrates can lead to negative side effects, like sub-acute ruminal acidosis (Enemark, 2008), which may disrupt the normal function of the ruminal ecosystem. While the additivity of energy from single feeds in compound feeds is commonly assumed in practical feed formulation, potential associative effects among feeds may impair the accurate compound feed formulation.

Potential associative effects among single feeds can be evaluated utilising GP procedures. One of the most commonly used procedures for determination of GP is HGT. In the present thesis, the *in vitro* GP of all feed samples was determined using HGT (Manuscript 2). Differences between calculated and observed GP at 24 h (**GP_{24}**) of compound feeds were negligible, with regression line slope close to 1 and high R^2 value of 0.97 (Figure 7). Therefore, the additivity of GP_{24} values was considered to be given.

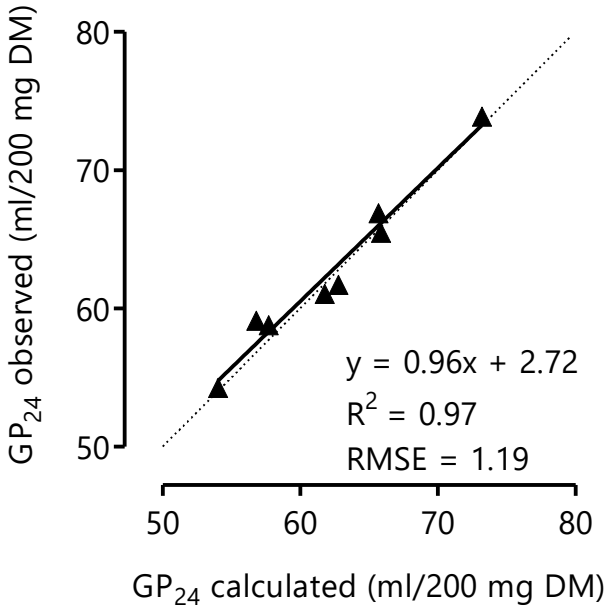

Figure 7. Comparison of calculated and observed GP_{24} values of compound feeds in mash form

Additivity of GP was previously evaluated in concentrate-forage combinations (Robinson *et al.*, 2009; Metzler-Zebeli *et al.*, 2012), where small associative effects existed only during early hours of incubation, and becoming negligible later. Differences in early hours of incubation may be the reason for numerically small associative effects regarding *c*GP, as calculated *c*GP deviated from observed in most

compound feeds (Figure 8), but with a maximum deviation of only 0.7%/h. The small associative effects on *c*GP were not reflected in GP_{24}.

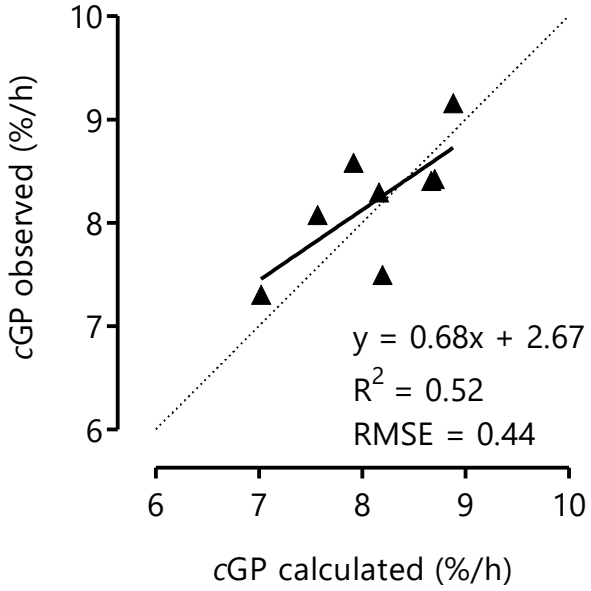

Figure 8. Comparison of calculated and observed *c*GP values of compound feeds in mash form

The mean difference between calculated and observed GP for all compound feeds was highest at 4 h of incubation (Table 3). However, this difference was only 2 ml/200 mg DM and was considered small. The calculated and observed *b*GP differed negligibly (Manuscript 2), and *b*GP was considered to be additive.

Table 3. Differences between calculated and observed gas production values of compound feeds at different incubation time points (h), expressed in % of observed values

Compound feed	2	4	6	8	12	24	48	72
1	13	13	4	3	2	1	1	1
2	21	17	11	3	0	0	0	0
3	11	12	4	3	-2	-2	0	0
4	12	6	0	-3	0	2	3	3
5	12	7	4	0	0	-2	0	0
6	11	6	4	3	2	2	3	3
7	11	12	4	0	0	0	2	2
8	11	12	4	3	2	4	5	5
average	13	11	4	1	1	1	2	2

Additivity of GP_{24} is important as GP_{24} can be used for calculation of **dOM** and ME. Equation of Menke and Steingass (1988) used for calculation of dOM from GP_{24} and proximate nutrients was presented in Manuscript 2. Therein, the GP_{24} values contribute largely to the predicted value of dOM, which amounted for samples of compound feeds in the present thesis from 67 to 79% of the dOM value (Annex 3).

Thus, the evaluation of additivity of dOM is closely related to the additivity of GP_{24}. Therefore, it was not surprising that differences between calculated and observed values of dOM did not exceed 2 pp (Figure 9). Thus, additivity of dOM was considered to be given.

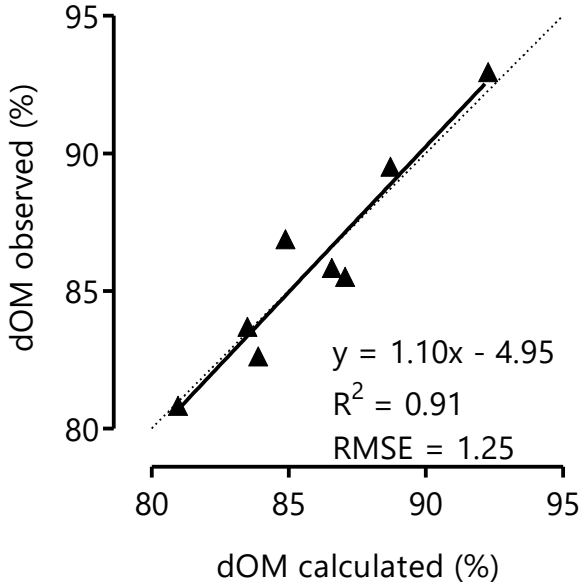

Figure 9. Comparison of calculated and observed dOM values of compound feeds in mash form

Determined GP_{24} values were also used together with proximate nutrients to estimate **ME values** of feed samples. As explained in Manuscript 2, equations for predicting ME values were typically developed for a specific feed or group of feeds. However, for the purpose of additivity calculation this could lead to big apparent deviation of calculated and observed values, as it was the case in the present thesis (Figure 10). The ME equations for both single feeds and feed mixtures were formerly developed by Menke and Steingass (1988) and GfE (2009). The equation of Menke and Steingass (1988) is described in Manuscript 2, where it was used for non-cereal feeds only (soybeans, soybean meal, rapeseed meal, sunflower meal, DDGS, and sugar beet pulp). Because that equation was developed using also compound feeds, an attempt was made to use it for all single and compound feeds (Figure 9; Annex 4). Still, calculated and observed ME of compound feeds were not similar. Finally, using the GfE (2009) equation for all

feed samples, differences between calculated and observed values were small (Manuscript 2).

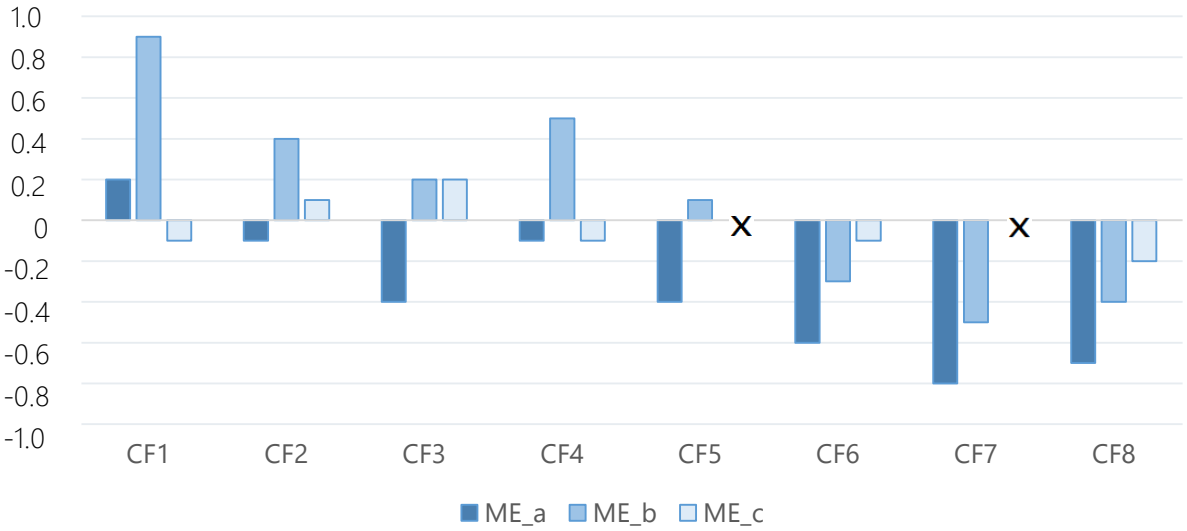

Figure 10. Differences of calculated (from single feeds) and observed ME values (MJ/kg DM) of compound feeds (CF1–8). Different equations for estimation of ME of single feeds were used: Krieg *et al.* (2017) or Menke and Steingass (1988) depending on the feed group (ME_a); Menke and Steingass (1988) for all single feeds (ME_b); GfE (2009) for all single feeds (ME_c). x indicates no difference between calculated and observed ME values.

The measured associative effects that were obtained when using different ME equations were therefore considered to be the result of calculation method rather than actual feed interactions. While feed and feed group specific ME equations result in higher accuracy of prediction for the respective feed, they cannot be used for routine prediction of ME of compound feeds. To accurately predict the ME values of compound feeds, ME equations developed for a wide variety of single and compound feeds are necessary.

The main source of energy in cereal grains is ST. Ruminal ST degradation characteristics are important not only in the energy evaluation, but also in some protein evaluation systems like the Dutch DVE/OEB-system (Tamminga *et al.*, 1994). Thus, accurate estimation of ruminal ST degradation from different feeds is necessary for optimal dairy cow feeding. While ST is known to be completely digested in ruminants (Offner and Sauvant, 2004), the extent of **ruminal ST degradation** and post-ruminal ST digestibility is known to vary among single feeds (Sutton, 1985; Cerneau and Michalet-Doreau, 1991). Therefore, compound feeds in the present thesis were expected to also have different ruminal ST degradation characteristics, reflecting degradation of single feeds contained therein.

In the present study, differences between calculated and observed $EDST_{IN\_SITU}$ values of compound feeds were not higher than 2 pp (Manuscript 1; Figure 11). The $EDST_{IN\_SITU}$ values for both single and compound feeds were overall high, and big difference between calculated and observed values was not expected. Because ST was the main energy source in examined compound feeds, additivity of ruminal ST degradation reinforces the conclusion that the additivity of ME values was given.

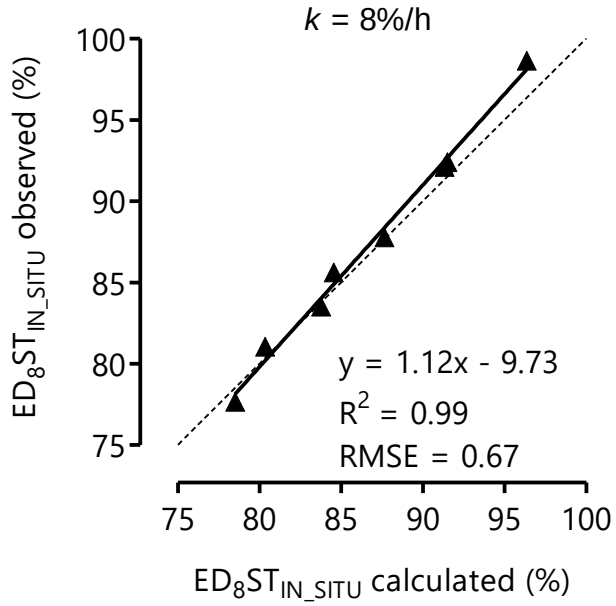

Figure 11. Comparison of calculated and observed $ED_8ST_{IN\_SITU}$ values of compound feeds in mash form

The evaluation of additivity of cST_{IN_SITU} was not possible in the present work. One anomaly was noted for cST_{IN_SITU} of wheat bran, where the estimate was extremely high (1748%/h; Manuscript 1). Because the aST_{IN_SITU} value of wheat bran was 80%, it was concluded that the *in situ* method is probably not suitable for evaluation of ST degradation of wheat bran when using bag pore size of 50 µm. In future studies, the reduced bag pore size may be suggested. However, incubation in bags with smaller pores is known to lead to gas accumulation within bags, which may affect degradation results (Seifried *et al.*, 2015). Cerneau and Michalet-Doreau (1991) used bag pore size of 46 µm, with the same sample size to bag surface area ratio as in the present thesis. They ground feed samples through a 0.8 mm screen which was smaller than 2 mm screen used in the present thesis. However, single feeds in the present work were also all ground through a 3 mm screens in the feed mill (as described in Chapter 5.1.1.), which may have further increased the fineness of feed particles. Cerneau and Michalet-Doreau (1991) fitted the ST disappearance as described by the equation of Ørskov and McDonald (1979), without using the lag phase unlike in the present

experiment. They estimated the $a\text{ST}_{\text{IN\_SITU}}$ fraction of wheat bran at 83%, which was even higher than the one determined in the present thesis by 3 pp, yet they estimated $c\text{ST}_{\text{IN\_SITU}}$ value that was considered more plausible (25.4%/h vs. 1748%/h). This could mean that the choice of equations for fitting the disappearance of ST to the curve had the most significant impact on the estimated $c\text{ST}_{\text{IN\_SITU}}$ between the present work and the work of Cerneau and Michalet-Doreau (1991). However, the fit of ST degradation parameters to the data in the present work was good both when using the model with and without lag phase (R^2 = 0.96, $s_{x.y}$ = 0.90), although the $c\text{ST}_{\text{IN\_SITU}}$ was even higher in the model with the lag phase. Wheat bran ST degradation should be further researched with multiple pore sizes with special attention to differences in ST disappearance equations, and also *in vitro*. Interestingly, even with such an extreme value of $c\text{ST}_{\text{IN\_SITU}}$ in one single feed, the additivity of $\text{EDST}_{\text{IN\_SITU}}$ was given. In the ED equation used (Manuscript 1):

$$\text{ED} = \text{a} + \left(\frac{\text{b} \cdot \text{c}}{\text{c} + \text{k}}\right) \cdot (\text{e}^{-\text{k} \cdot \text{lag}})$$

the difference in *c* does not have a large influence on the ED value. When the $c\text{ST}_{\text{IN\_SITU}}$ value from the Cerneau and Michalet-Doreau (1991) is used instead of the one determined in the present study, the difference in $\text{ED}_8\text{ST}_{\text{IN\_SITU}}$ value for wheat bran is relatively small (94% vs. 98%). Such small difference in only one single feed could not affect the $\text{ED}_8\text{ST}_{\text{IN\_SITU}}$ values of compound feeds containing wheat bran (1, 2, 3, and 7), and was considered to be not relevant for the purpose of evaluation of additivity. Therefore, the extreme wheat bran $c\text{ST}_{\text{IN\_SITU}}$ value was used for calculation of EDST values and evaluation of additivity in the present thesis. However, the ruminal ST degradation of wheat bran may be a specific case in this regard, since it had a very high $a\text{ST}_{\text{IN\_SITU}}$ value, and small $b\text{ST}_{\text{IN\_SITU}}$, and also the inclusion level of wheat bran in compound feeds did not exceed 15% of DM. For other single feeds the influence of potential extreme values of $c\text{ST}_{\text{IN\_SITU}}$ on $\text{EDST}_{\text{IN\_SITU}}$ may be more relevant.

Ruminal ST degradation was previously found to correlate well with *in vitro* GP characteristics for starch-rich feeds, and this could alleviate the necessity for conducting time-consuming *in situ* studies. Seifried *et al.* (2016) have found an acceptable **prediction of $c\text{ST}_{\text{IN\_SITU}}$ from *c*GP** in maize samples. Similar approach was attempted for all single feeds in the present thesis in which ST degradation was evaluated, exclusive of wheat bran that had an extremely high $c\text{ST}_{\text{IN\_SITU}}$ value; and also for compound feeds. While a fair prediction in single feeds was possible (Figure 12), no prediction was possible for compound feeds (Figure 13), presumably due to ST concentration of compound feeds being vastly different (from 18.7 to 48.6% in DM), as ST was not the main carbohydrate in all single feeds.

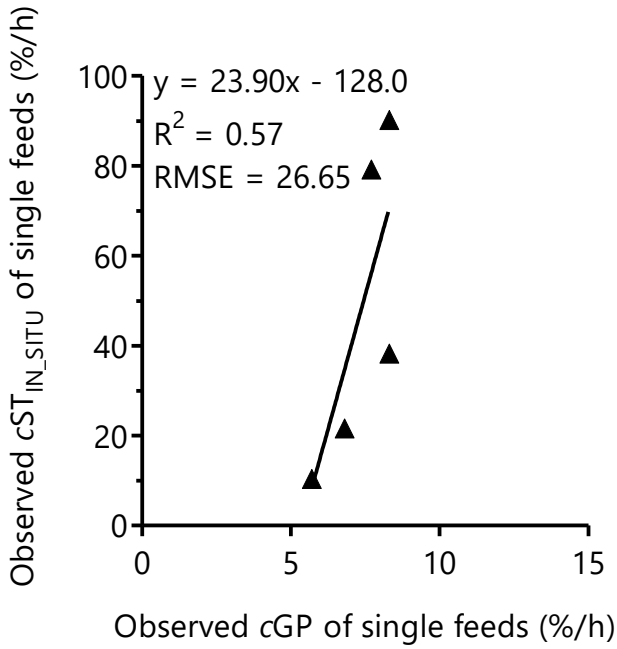

Figure 12. Prediction of cST$_{IN_SITU}$ from cGP values in samples of single feeds

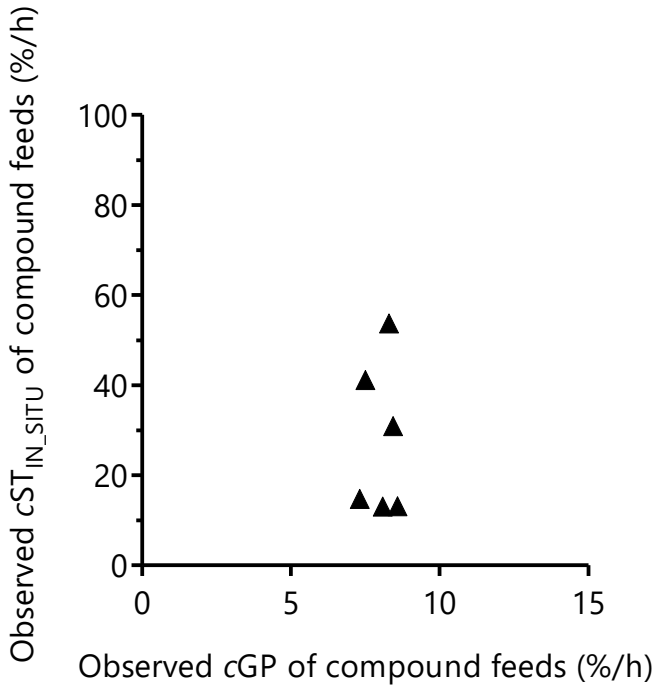

Figure 13. Comparison of cST$_{IN_SITU}$ and cGP values in samples of compound feeds

Therefore, prediction of cDM$_{IN_SITU}$ from cGP was attempted, assuming that precision of prediction for the DM of feed samples would be more accurate. However, the accuracy

of prediction in single feeds was low (Figure 14), and prediction for compound feeds was not possible (Figure 15). Perhaps, the variation of values was too low. It was concluded that the *in situ* ST and DM degradation characteristics could not be accurately predicted from GP characteristics across single feeds, and also for compound feeds in the present thesis.

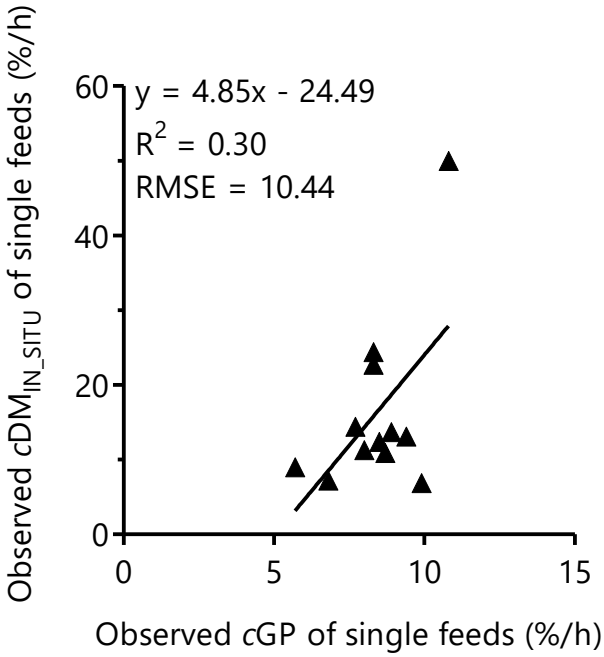

Figure 14. Prediction of cDM$_{IN_SITU}$ from cGP values of single feeds

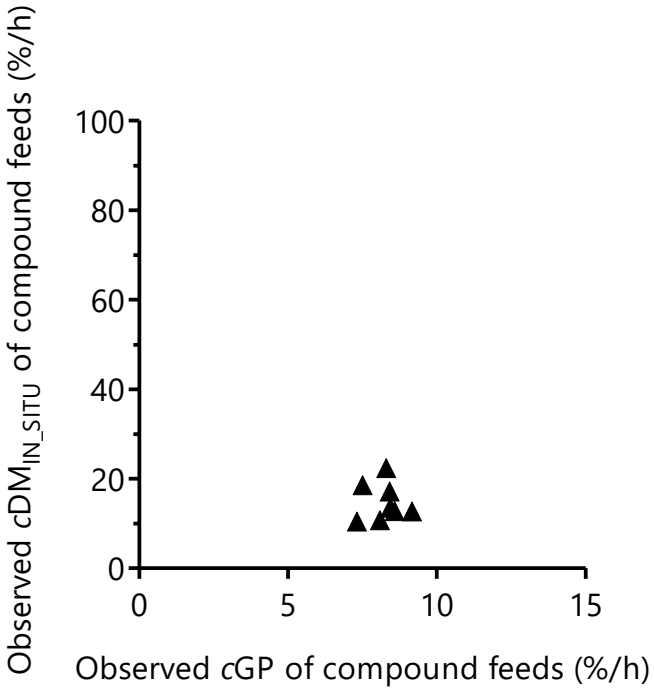

Figure 15. Comparison of cDM$_{IN_SITU}$ and cGP values of compound feeds

The overview of calculated and observed energy values and related values is given in Annex 2b. The highest RMSE values relative to the mean observed values were found for individual *in situ* degradation parameters of ST, for cST_{IN_SITU} at 199%, and $lagST_{IN\_SITU}$ at 71%. This is related to issues of measuring the cST_{IN_SITU} of some single feeds *in situ*, as discussed previously in this chapter.

Overall, additivity of energy and related values was considered to be given in compound feeds used in the present thesis. However, special attention has to be given to the choice of equations for estimation of ME of single feeds when evaluating additivity of ME values of single feeds in compound feeds.

5.3. Effects of pelleting on ruminal degradation of nutrients and feeding values of compound feeds

Processing of animal feed can be chemical or physical (Campling, 1991). Physical methods such as rolling or grinding disturb the grain structure allowing microbes and digestive enzymes an easier access to feed nutrients. Heating causes gelatinisation of ST (Collison and Chilton, 1974). Processing can change the rate and extent of both CP and ST degradation in the rumen, and can shift the digestion from rumen to intestines (Razzaghi *et al.*, 2016). Pelleting is a common method of compound feed processing combining both physical force and heat (Thomas and van der Poel, 1996). Pelleting has the benefits of better feed nutrient homogenisation, increased hygienic quality, easier transport and storage compared to compound feeds in mash form (Thomas and van der Poel, 1996; Thomas *et al.*, 1997). During the pre-treatment (conditioning) of compound feeds just before pelleting, they are exposed to increased temperature or addition of water (commonly in the form of steam). For a more detailed review of effects of pelleting on CP and ST, the reader is referred to Svihus and Zimonja (2011).

5.3.1. Effects of pelleting on protein values of compound feeds

Heat, moisture, and shear forces are the main causes of protein denaturation, and are all part of the pelleting process (Thomas *et al.*, 1998). The protein denaturation temperature is closely related to the moisture content, and is known to differ among feeds. During high heat and low moisture condition, irreversible protein cross-linking and Maillard reaction can occur. The Maillard reaction represents an interaction between carbohydrates (in particular reducing sugars fructose, glucose, and pentose) and proteins (in particular free amino groups from lysine). High level of ST in single feeds increases the risk of Maillard reaction. The Maillard reaction usually results in better pellet binding, but also lowers availability of some nutrients (Thomas *et al.*, 1998). Pelleting is known to shift the degradation of protein in ruminants from rumen to the intestines, by increasing the RUP fraction of feed CP (Svihus and Zimonja, 2011). However, excess heat during processing can negatively affect intestinal digestion of protein (Satter, 1986). Any effect of pelleting is not the same among single feeds, not even within feed groups (Aguilera *et al.*, 1992). Because processing (and heat) affects the protein value differently among single feeds, knowledge of effects of pelleting on compound feeds containing a wide variety of protein sources is important.

In the present thesis, **ruminal degradation of CP** was examined in compound feeds in mash and pelleted form using the *in situ* procedure. Pelleting significantly increased the $EDCP_{IN\_SITU}$ in three compound feeds, but in the other five compound feeds no

significant difference was found (Manuscript 1). The magnitude of effects of pelleting varied among compound feeds, which resulted in small accuracy of regression equation (Figure 16). Results from the present thesis contrasted to Razzaghi e*t al*. (2016), who found that pelleting lowered $EDCP_{IN\_SITU}$ in all binary feed mixtures tested in their study.

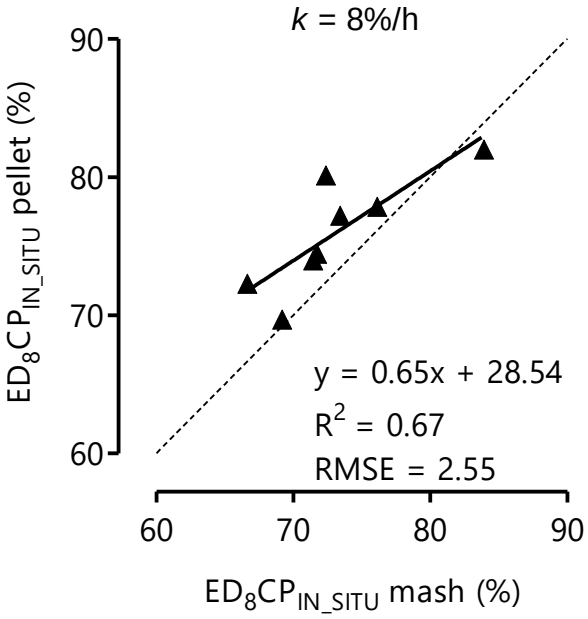

Figure 16. Comparison of $ED_8CP_{IN\_SITU}$ values of compound feeds in mash and pelleted form

Decrease in ruminal CP degradation and shifting the site of digestion to the intestine can occur when heat applied during feed processing is high. With a pellet exit temperature of 80–90°C in the present work this may not have been the case. A possible explanation for the increase of $EDCP_{IN\_SITU}$ values in some compound feeds was related to the change in particle size distribution. Pelleting increases the share of fine feed particles (Abdollahi *et al*., 2011), leading to higher washout of undegraded particles from *in situ* bags (Goelema *et al*., 1999). In the present work, results of particle size determination showed that the share of feed particles passing through smaller sieves (sieve size 0.5 mm and less) was higher for pelleted compound feeds than mash feeds (Manuscript 1). The aCP_{IN_SITU} fraction was increased after pelleting in most compound feeds, with a maximum of 13 pp in compound feed 8. The increase of fine particles was uneven among compound feeds. For example, share of particles passing the 0.125 mm sieve increased from 1 pp in compound feed 1 to 11 pp in compound feed 5. At the same time, $ED_8CP_{IN\_SITU}$ values were increased in those compound feeds by 1 and 5 pp, respectively. This difference in the extent of particle size change among compound feeds may stem from single feeds. It was previously shown that for example

in barley the share of particles smaller than 0.2 mm during pelleting was decreased more readily than in wheat (Rakić, 2012). The compound feeds in the present study differed in single feed composition, and thus pelleting lowered particle size in different compound feeds unevenly. However, in mash compound feed 4, share of particles that passed the 0.125 mm sieve was increased by only 5 pp, yet the increase in $ED_8CP_{IN\_SITU}$ was the highest among compound feeds at 8 pp. Presumably, the change in the particle size may not be the only cause of $EDCP_{IN\_SITU}$ increase in some compound feeds. It is possible that in some compound feeds small associative effects may have occurred, however the particle size reduction is still a major contributor to an increase in $EDCP_{IN\_SITU}$. Reduction in particle size can increase the surface area of nutrients like CP available to digestive enzymes (Goodband *et al*., 1995). This could lead to higher cCP_{IN_SITU} as a result of pelleting, but in the present work pelleting had no significant effect on cCP_{IN_SITU}. This was in agreement with D'Mello (2000), who found no such mechanism in feed samples tested with the *in situ* procedure, as described in Chapter 5.1.1. Finally, it was concluded that the measured differences between $EDCP_{IN\_SITU}$ of mash and pelleted compound feeds were mostly a consequence of particle size alteration, and that pelleting has only a small effect on ruminal CP degradation of compound feeds, under the conditions used in the present work.

Results of the *in situ* study showed that the RUP supply to the duodenum from dietary CP was unaffected by pelleting. Since **uCP** consists of RUP and MCP, pelleting was expected to have only a small effect on uCP concentration. The *in vitro* studies like eHGT were less affected by particle size differences between mash and pelleted compound feeds, since feed samples are incubated inside glass syringes together with rumen fluid and buffer solution. However, an increased microbial population density around smaller feed particles may affect the rate of fermentation *in vitro* (Gerson *et al*., 1988), which may possibly increase the non-ammonia-N concentration, which is used to estimate uCP in eHGT (Steingaß and Südekum, 2013). In the present study, pelleting led to only a slight increase of the uCP concentration in compound feeds with lower CP concentration, and slight decrease of the uCP concentration in compound feeds with higher CP concentration. Even though the regression line slope was not close to 1, due to only small numerical differences between uCP of mash and pelleted compound feeds, it was concluded that pelleting had no large effect on uCP values (Figure 17; Manuscript 2). Differences in uCP concentration between mash and pelleted compound feeds did not exceed 24 g/kg DM. This was probably due to relatively low heat produced during pelleting. Application of heat at 105°C and above was shown to increase the uCP concentration in legumes (faba beans, lupins, and peas) when incubated together with grass silage and barley (Vaga *et al*., 2017). The extent of

uCP increase after more intense heating in mixtures of single concentrate feeds is not known. However, it is probably closely related to the extent of the increase in RUP.

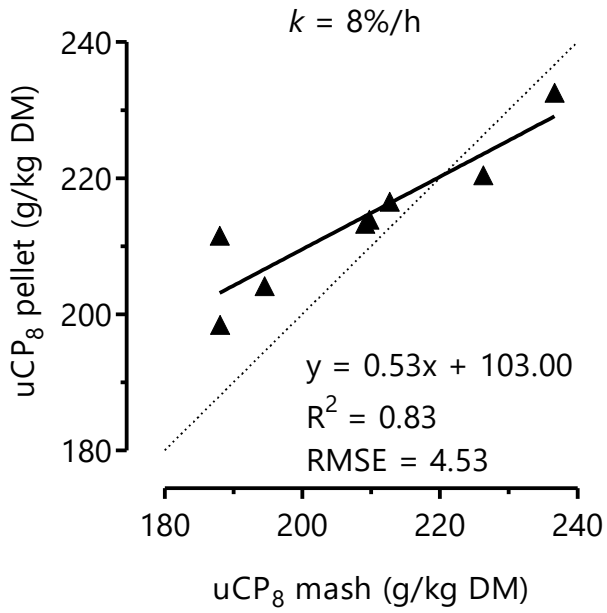

Figure 17. Comparison of uCP_8 values of compound feeds in mash and pelleted form

In high-yielding dairy cows the continuous supply of high quality RUP with high intestinal digestibility is necessary (Calsamiglia and Stern, 1995). Since compound feeds are often fed in pelleted form, the effect of pelleting on ID_{RUP} of compound feeds should be understood, however this was previously not reported. Studies on effects of pelleting on **ID_{RUP}** of single feeds are scarce, and previously the usage of fistulated cows for the mobile bag technique was necessary. In the present thesis, the less work-intensive three-step enzymatic method of Calsamiglia and Stern (1995) was used for estimation of ID_{RUP}. After 16 h *in situ* incubation, feed samples were incubated in pepsin-pancreatin solutions *in vitro*. The ID_{RUP} was lowered by pelleting in all compound feeds except for the compound feed 1, with a maximum of 15 pp decrease in compound feed 3 (Figure 18). This indicates that the effect of heat produced during the pelleting procedure, though small and hardly relevant in the rumen, still had a noticeable negative effect on intestinal digestibility. Since the level of heat used in the present work was low, it would be hard to substitute the currently used industrial pelleting process. Some other feed processing procedures like extrusion, while using higher heat levels, showed no negative effects on ID_{RUP} of peas (Walhain *et al*., 1992), and even increased the ID_{RUP} of protein-rich feeds like soybeans, lupins and peas (Solanas *et al*., 2008), as determined in the mobile bag trials. Further research should examine the effects of different types of feed processing on compound feeds.

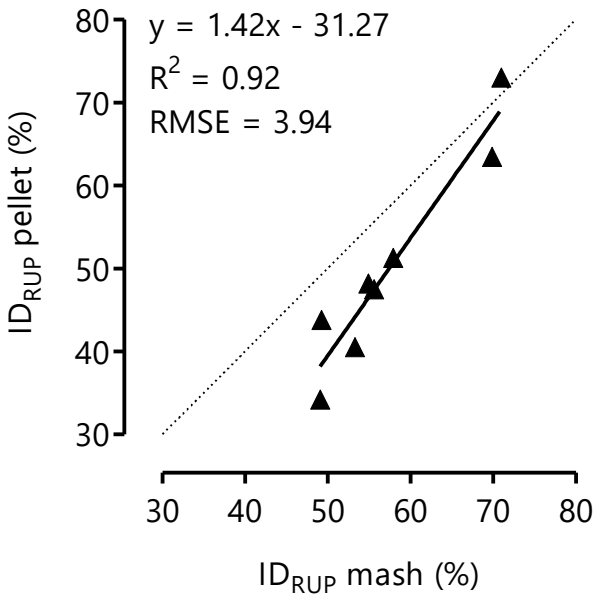

Figure 18. Comparison of ID_{RUP} values of compound feeds in mash and pelleted form

Alternatively, differences between ID_{RUP} of mash and pelleted compound feeds may be explained with particle size loss through bag pores during the *in situ* part of the procedure. Similarly to the particle disappearance in the main *in situ* study (Manuscript 1), CP disappearance was higher in pelleted compound feeds when compared to mash (Figure 19), even though samples were incubated only for one time point (16 h). It is not known if the CP that potentially left the *in situ* bags due to the particle size change differs in its intestinal digestibility from the CP that remained within the bags. This may affect results of the three-step method. This issue is particularly problematic in studies evaluating effects of pelleting or similar processing procedure that leads to particle size change. For such trials, this methodological constraint may be circumvented by using an *in vitro* step instead of *in situ* step for simulation of ruminal degradability (Gargallo *et al.*, 2006; Irshaid, 2007). Unfortunately, validation of ID_{RUP} using the mobile bag technique would presumably suffer from same methodological errors related to the disappearance of particulate matter through bag pores, similarly as experienced during *in situ* studies.

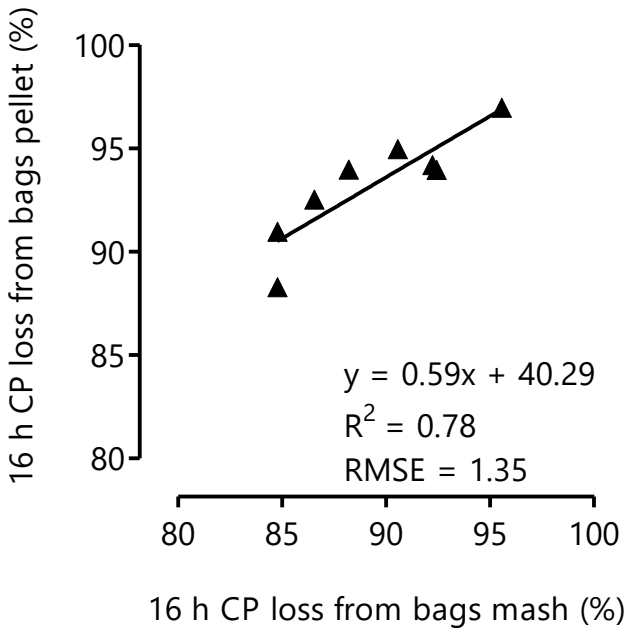

Figure 19. Comparison of CP disappearance from *in situ* bags at 16 h of incubation from compound feeds in mash and pelleted form

Because pelleting reduced the ID_{RUP} in most compound feeds, the CP fraction C in the **CNCPS** model should have been affected, since it represents the fraction unavailable for degradation in the rumen or digestion in intestines, consisting of N associated with lignin and products of Maillard reaction (Sniffen *et al.*, 1992; Licitra *et al.*, 1996; Chrenková *et al.*, 2014). While the share of C fraction in pelleted compound feeds did not completely correspond to the one in mash (as indicated by R^2 value of 0.68), the only notable increase in C fraction due to pelleting was found in compound feed 6 (2.5% vs. 5.4%), and only a small difference was found in other compound feeds (Figure 20). As this deviation was found only in one compound feed, the likely cause was the composition of compound feed, but no specific single feed can be related to it.

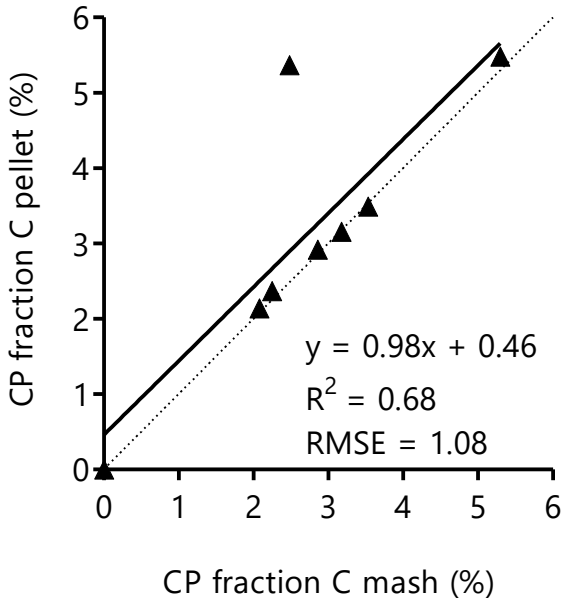

Figure 20. Comparison of the share of CP fraction C in compound feeds in mash and pelleted form

All other CP fractions (A, B1, B2, B3) corresponded well between mash and pelleted compound feeds, resulting in slopes of the regression close to 1, intercepts close to 0 (except for B2 fraction) and R^2 values of 0.86 and above (Manuscript 2). It was concluded that pelleting did not influence CP fractions of compound feeds, and numerical differences among CP fractions of compound feeds in mash and pelleted form were considered to be negligible for practical purposes.

Overall, it can be concluded that pelleting had only a small influence on protein values. This is probably caused by the relatively modest heat applied during the processing of compound feeds that did not affect nutrient structure within compound feeds, and thus did not affect the $EDCP_{IN\_SITU,}$ uCP concentration, and the CP fractions of compound feeds. However, pelleting lowered ID_{RUP} of most compound feeds.

5.3.2. Effects of pelleting on energy values and related values of compound feeds

In the present thesis, the *in vitro* determined **GP_{24}** did not differ more than 2.4 ml/200 mg DM between mash and pelleted compound feeds (Manuscript 2). Even though the regression slope line was only 0.82, the R^2 value was high (0.97; Figure 21), and the numerical differences between GP_{24} of mash and pelleted compound feeds were considered to be negligible. Therefore, it was concluded that pelleting had no effect on GP_{24}.

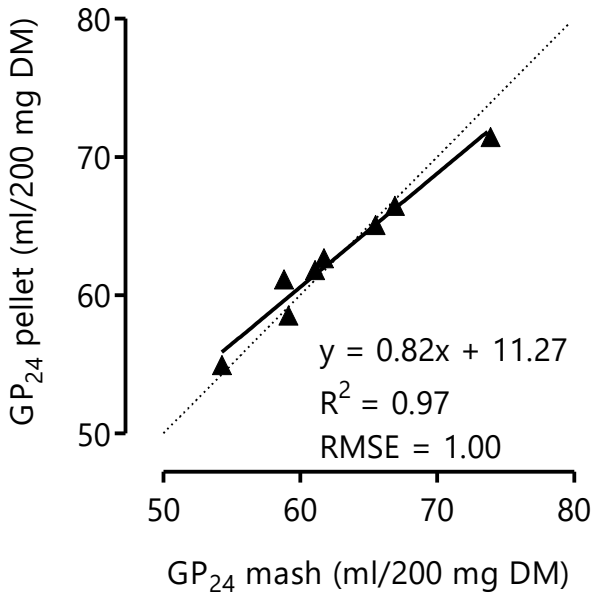

Figure 21. Comparison of GP_{24} values of compound feeds in mash and pelleted form

However, *c*GP was higher in all pelleted compound feeds when compared to mash (Figure 22). This may be a consequence of particle size change, as pelleted compound feeds had a higher share of smaller particles. This may increase the availability of feed particles to microbes in the closed *in vitro* system in the first hours as indicated by increased *c*GP in pelleted compound feeds, without affecting the maximum GP (*b*GP) (Manuscript 2). Therefore, no change in GP_{24} due to pelleting was detected. Although lowering the feed particle size has a greater effect on GP increase in forages than readily degradable feeds, this mechanism should not be ruled out for some concentrate feeds (Rymer *et al.*, 2005).

Some types of feed processing such as extrusion generate a significant amount of heat. Extrusion (typically 80 to 200°C) was shown to increase the gelatinisation of ST in cereals, leading to higher *b*GP and *c*GP (Solanas *et al.*, 2008). Modest heat produced during pelleting in the present study was probably not enough to achieve high levels of ST gelatinisation that would affect GP characteristics.

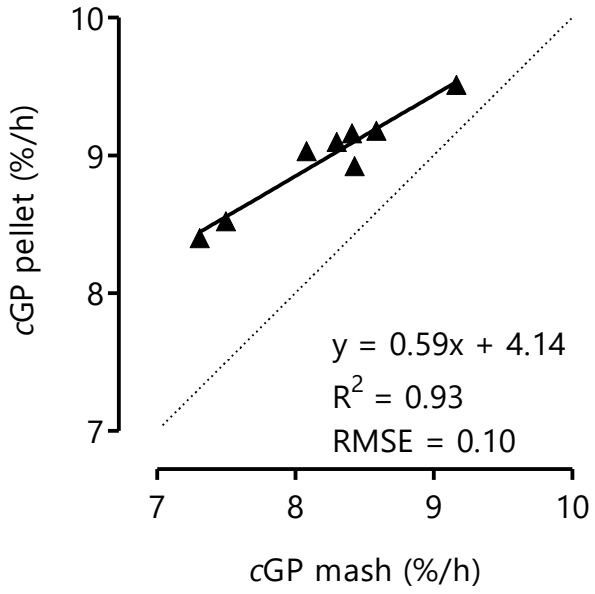

Figure 22. Comparison of *c*GP values of compound feeds in mash and pelleted form

As explained in Chapter 5.2.2., GP_{24} value is used to calculate **dOM**, and contributes significantly to its value. Thus, effect of pelleting on dOM of compound feeds was expected to be negligible. Significant differences of slope from 1 and intercept from 0 were found for comparison of dOM between mash and pelleted compound feeds, but numerical differences did not exceed 2 pp (Figure 23; Manuscript 2). Such a difference was considered to be negligible for practical purposes, and pelleting was considered to have no effect on dOM of compound feeds.

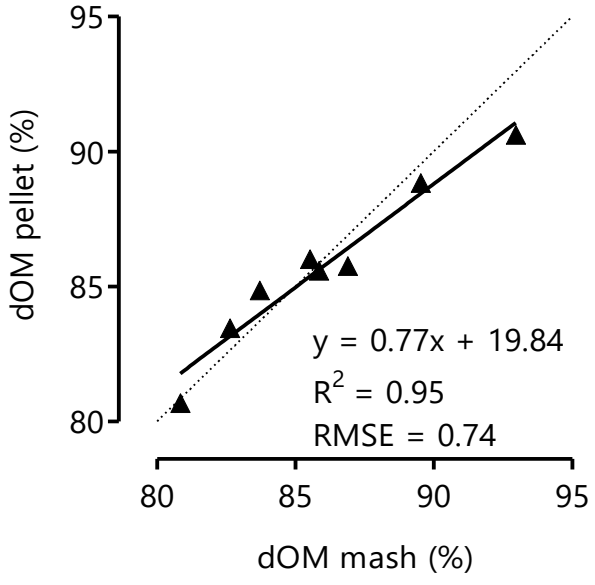

Figure 23. Comparison of dOM values of compound feeds in mash and pelleted form

Similarly as dOM value, **ME** is also calculated using GP_{24} values. Regression between ME of mash and pelleted compound feeds resulted in significant differences of CI ranges for slope from 1 and intercept from 0. However, numerical differences between ME of mash and pelleted compound feeds were not greater than 0.3 MJ/kg DM (Figure 24; Manuscript 2), which was considered to be not important for practical feed formulation, and that ME concentration is unaffected by pelleting.

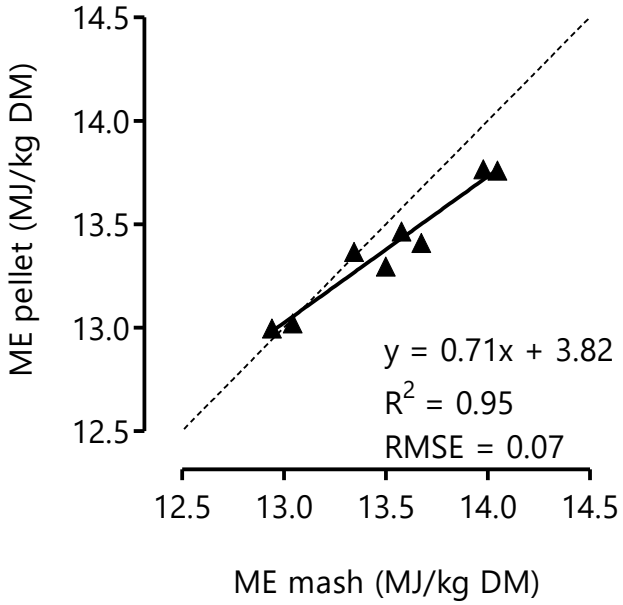

Figure 24. Comparison of ME values of compound feeds in mash and pelleted form

The processing of compound feeds is known to have an effect on gelatinisation of ST (Svihus *et al.*, 2005), leading to greater **ST degradation in the rumen** with the help of ruminal amylolytic microorganisms, and release of energy that can be utilised to support microbial growth. However, too high ST degradation in the rumen can have negative consequences such as induction of acidosis. Thus, for accurate feeding of high-producing dairy cows, precise information on the influence of pelleting on ST degradability in compound feeds is necessary. In the present work, the $EDST_{IN\_SITU}$ values did not differ between mash and pelleted compound feeds by more than 4 pp (Figure 25; Manuscript 1). Effective ruminal degradation of ST for k = 8%/h was overall high both in mash (between 78 and 99%) and pelleted (between 82 and 97%) compound feeds.

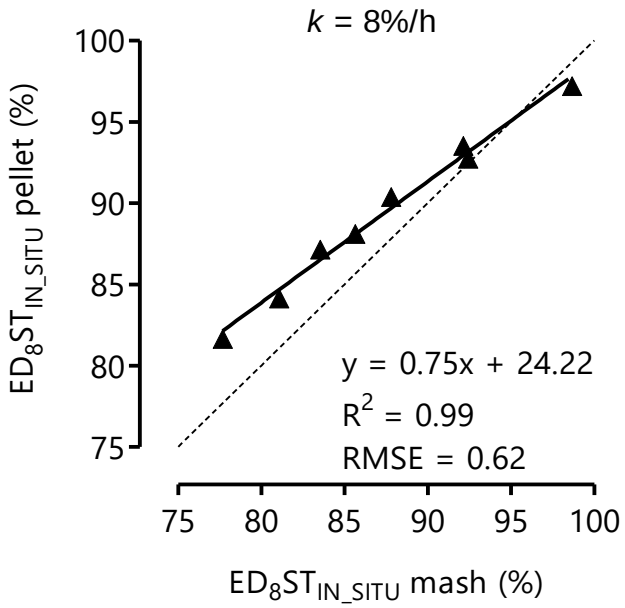

Figure 25. Comparison of $ED_8ST_{IN\_SITU}$ values of compound feeds in mash and pelleted form

Effects of processing on ST differ among single feeds, and the effect is greater in feeds with slower ST degradation (Offner *et al*., 2003). Different processing procedures of compound feeds are known to have different influence on ruminal ST degradation (Nocek and Tamminga, 1991). Gelatinisation of ST is mainly dependent on the moisture content of feeds and the heat produced during processing (Lund and Lorenz, 1984). The moisture content and heat produced during pelleting in the present thesis was presumably not high enough to enable significant ST gelatinisation in most of the ST-rich single feeds contained within compound feeds. Svihus *et al*. (2004) reported only low extent of ST gelatinisation after pelleting diets for poultry. The minimal moisture content range required for ST gelatinisation is 30 to 50% at the temperature of minimum 80–90°C, depending on the feed (Collison and Chilton, 1974; Olkku and Rha, 1978; Wood, 1987). Because the moisture of compound feeds in the present work did not exceed 11%, it is unlikely that ST gelatinisation would occur. Lowering of $EDST_{IN\_SITU}$ as a result of pelleting is probably rather methodologically linked to the lowering of $EDCP_{IN\_SITU}$, mainly caused by the change in feed particle sizes and feed particles leaving the bags undegraded (see Chapter 5.3.1.). Tothi *et al.* (2003) found that expander processing increased the aST_{IN_SITU} of maize and barley when incubated in bags with pore sizes of 15 or 36 µm, and that the decrease was greater for the higher pore size, and related to change in feed particle size. In the present work, the aST_{IN_SITU} of most pelleted compound feeds was increased when compared to

mash, up to 13 pp, using the bag pore size of 50 µm. However, similarly to Tothi *et al.* (2003), the effect on $EDST_{IN\_SITU}$ was small.

Overall, it was concluded that the GP_{24}, dOM, ME, and $EDST_{IN\_SITU}$ were not influenced by pelleting in the present thesis. While results indicated deviations between mash and pelleted compound feeds, numerical differences were in most cases small. The reason for this is presumably the relatively low intensity of heat produced during pelleting (up to 80–90°C) in combination with the low moisture content of compound feeds (up to 11%) that did not lead to structural change of ST.

5.4. Additivity of ruminal degradation of nutrients and feeding values of single feeds in pelleted compound feeds

In the Chapter 5.3. it was concluded that the effect of pelleting on feeding value of compound feeds was overall small. Compound feeds are commonly fed to dairy cows in pelleted form. Therefore, the potential existence of associative effects among single feeds in pelleted compound feeds are of concern for practical feed formulation, yet the knowledge of such effects in not sufficient. Protein degradation characteristics and energy and related values of compound feeds calculated from single feeds were presented in Chapter 5.2. and in Manuscripts 1 and 2. In this chapter, previously calculated values for compound feeds were compared with observed values for pelleted compound feeds. Comparison of calculated and observed values for pelleted compound feeds was performed using simple linear regressions.

5.4.1. Additivity of protein values of single feeds in pelleted compound feeds

Calculated and observed values of pelleted compound feeds related to CP degradation are presented in Table 4. The **$EDCP_{IN\_SITU}$ values** of pelleted compound feeds could be accurately calculated from single feeds, and thus values were considered to be additive in pelleted compound feeds both for k = 5 and 8%/h. However, individual CP degradation parameters were not considered to be additive. Slopes of regression lines were significantly different from 1 for values of cCP$_{IN_SITU}$, and lagCP$_{IN_SITU}$ and intercepts were significantly different from 0 for values of cCP$_{IN_SITU}$. While for aCP$_{IN_SITU}$ slope and intercept were not significantly different from 1 and 0 respectively, the slope of 0.37, R^2 of 0.16, and high RMSE value of 4.61 indicated very low accuracy of prediction of aCP$_{IN_SITU}$ in pelleted feeds from single feeds. Similarly, low accuracy of prediction of bCP$_{IN_SITU}$ in pelleted feeds from single feeds was found.

The effect of pressure toasting (3 min at 132°C) on mixtures of peas, lupins and faba beans was tested by Goelema *et al*. (1998). They found that the CP degradability of treated mixtures could be calculated from single feeds. These researchers used the bag pore size of 40 µm, which was smaller than 50 µm used in the present work. This, combined with grinding of feed samples on a larger sieve mesh size than in the present work (3 vs. 2 mm), may have lowered the particle loss from the bags in their work. In the present work, the 50 µm pore size was chosen because smaller pore sizes may lead to gas formation within bags in samples of cereal grains (Seifried *et al*., 2015) which were contained in compound feeds in the present work, but not in mixtures of Goelema *et al*. (1998).

In Chapter 5.3. it was mentioned that pelleting reduced the particle size of compound feeds. The extent of particle size reduction may not be equal among single feeds contained therein. This would presumably be mirrored in different increase of particle

loss in pelleted compound feeds, depending on the single feed composition, and leading to associative effects in different compound feeds. However, Svihus *et al*. (2004) found that pelleting reduced the differences in particle size distribution between different poultry diets. In the present work, the range of particles passing through 0.5 mm sieve was 87–92% in mash and 91–94% in pelleted compound feeds, and the range of particles passing through 0.125 mm sieve was 65–71% in mash and 73–77% in pelleted compound feeds (Manuscript 1), which is in agreement with Svihus *et al*. (2004). This means that particle size contributed equally to particle loss through bags during *in situ* study, and that differences in the extent of associative effects in each pelleted compound feed were presumably more related to the single feed composition.

Table 4. Results of simple linear regressions for calculated and observed ruminal degradation characteristics and feeding values of pelleted compound feeds

	Slope	Slope CI	Intercept	Intercept CI	R^2	RMSE
$ED_5CP_{IN\_SITU}$	0.87	0.52 to 1.22	11.96	-15.72 to 39.65	0.86	1.25
$ED_8CP_{IN\_SITU}$	0.85	0.52 to 1.19	12.82	-12.00 to 37.64	0.87	1.62
aCP_{IN_SITU}	0.37	-0.49 to 1.23	26.84	-6.43 to 60.11	0.16	4.61
bCP_{IN_SITU}	0.51	-0.23 to 17.72	26.17	-17.18 to 69.52	0.32	4.38
cCP_{IN_SITU}	1.85	1.02 to 2.68	-21.72	-38.63 to -4.813	0.83	2.59
$lagCP_{IN\_SITU}$	0.21	-0.38 to 0.79	0.06	-0.70 to 0.82	0.11	0.30
uCP						
k = 5%/h	0.41	-0.07 to 0.89	110.9	18.96 to 202.9	0.42	4.98
k = 8%/h	0.50	0.22 to 0.79	105.2	43.54 to 167.0	0.76	5.47
ID_{RUP}	1.42	0.38 to 2.45	-24.22	-79.14 to 30.69	0.65	8.00
CNCPS						
A	1.32	0.71 to 1.93	-1.85	-9.15 to 5.45	0.82	2.48
B1	1.54	0.68 to 2.41	-11.41	-25.79 to 2.98	0.76	3.26
B2	0.88	0.56 to 1.19	-10.21	-10.51 to 28.44	0.89	2.05
B3	1.63	0.86 to 2.39	-4.91	-10.65 to 0.83	0.82	1.39
C	0.68	-0.17 to 1.53	0.75	-2.49 to 3.98	0.39	1.50
ED_{CNCPS}						
k = 5%/h	0.12	-1.52 to 1.76	72.92	-48.13 to 194.00	<0.01	3.37
k = 8%/h	0.38	-1.27 to 2.03	50.77	-58.48 to 160.00	0.05	4.33

uCP = utilisable crude protein for ruminal passage rates (*k*) of 5%/h and 8%/h; ID_{RUP} = intestinal digestibility of rumen undegraded protein; CNCPS = Cornell Net Carbohydrate and Protein System with fractions: A = non-protein nitrogen; B1 = rapidly degradable true protein; B2 = moderately degradable true protein; B3 = slowly degradable true protein, C = undegradable and indigestible true protein; ED_{CNCPS} = effective protein degradation for ruminal passage rates of 5%/h and 8%/h, calculated using Fox *et al*. (2003). RMSE = root mean square error.

The **uCP** concentration of pelleted compound feeds could not be calculated from single feeds with good accuracy. Numerical difference between calculated and observed values was in most compound feeds small, but in some big differences existed, up to 19 pp in compound feed 1. Slope and intercept were significantly

different from 1 and 0 respectively, both for *k* = 5 and 8%/h. The additivity of uCP concentration was shown to be given for compound feeds in mash form (Chapter 5.2.1.), and pelleting had only a small effect on uCP (Chapter 5.3.1.). Therefore it was surprising that the additivity of uCP in pelleted compound feeds was not given. One reason for such difference could be the decreased particle size in pelleted compound feeds which may allow for easier access of digestive enzymes (Goodband *et al*., 1995), and facilitate quicker *in vitro* degradation of feed samples when compared to single feed. However, uCP concentration of all pelleted compound feeds was lower than calculated, except for compound feed 1. It is possible that heat produced during pelleting affected single feeds differently, and therefore affected the accuracy of calculation of uCP of pelleted compound feeds from single feeds.

Calculated and observed values of the **ID_{RUP}** differed non-systematically. The ID_{RUP} was underestimated for up to 5 pp in compound feed 4, and overestimated for up to 12 pp in compound feed 5. Although slope and intercept of the regression line were not significantly different from 1 and 0 respectively, the accuracy of prediction was low (R^2 = 0.65) and RMSE was high (8.00). Thus, the additivity of ID_{RUP} of single feeds to pelleted compound feeds was not given. This is different to the results of study from Goelema *et al*. (1998), although mixtures of only three single feeds were used in their study. The non-additivity of C fraction of CNCPS may be related to this issue.

For all **CP fractions**, slope and intercepts did not significantly deviate from 1 and 0 respectively. However, while R^2 values were higher than 0.80 for A, B2, and B3 fractions, fractions B1 (R^2 =0.76) and particularly C (R^2 =0.39) had a low accuracy of prediction. Fraction C is related to insoluble protein that cannot be degraded nor digested (Licitra *et al*., 1996), and this could be a result of feed processing treatment (Chrenková *et al*., 2014). However, numerical differences between calculated and observed CP fractions were in most cases negligible. When comparing ED_{CNCPS} values calculated using equations by Fox *et al*. (2003) and observed $EDCP_{IN\_SITU}$ values for pelleted compound feeds for both *k* = 5 an 8%/h, big deviations occured. It was concluded that the CNCPS model could not be used for accurate calculation of EDCP values in pelleted compound feeds, and that additivity of CP fractions was given.

In this chapter it was shown that the additivity of single feeds in pelleted compound feeds was given for $EDCP_{IN\_SITU}$ and CP fractions, but for uCP and ID_{RUP} values the additivity was not given.

5.4.2. Additivity of energy values and related values of single feeds in pelleted compound feeds

Comparison of calculated and observed values of pelleted compound feeds for energy values and related values are presented in Table 5. For *b*GP, *c*GP, and **GP_{24}** the slopes and intercepts were significantly different from 1 and 0 respectively, but the R^2 values for *b*GP and GP_{24} were high at 0.96. Also, numerical differences between calculated and observed values of *b*GP and GP_{24} were considered to be small, not more than 4 ml/200 mg DM for *b*GP and 3 ml/200 mg DM for GP_{24}. This lead to the conclusion that *b*GP and GP_{24} of pelleted compound feeds could be accurately predicted from single feed values. Calculated *c*GP values underestimated the observed *c*GP values in all pelleted compound feeds, but this underestimation was uneven, and this resulted in small accuracy of prediction (R^2 = 0.40). In Chapter 5.3.2. it was discussed how the likely culprit for the increase of *c*GP in pelleted compound feeds was the lowering of particle size due to pelleting, allowing greater surface for microbial degradation, which was especially relevant during early hours of incubation. As discussed in Chapters 5.2.2. and 5.3.2., the GP_{24} is used to calculate **dOM** and **ME**. The ME of single and compound feeds was calculated using the same equation of GfE (2009). Because only small differences between calculated and observed values of GP_{24} were found, this was also mirrored for dOM and ME. It was concluded that dOM and ME of pelleted compound feeds can be accurately predicted from single feeds.

Table 5. Results of simple linear regressions for calculated and observed ruminal degradation characteristics and feeding values of pelleted compound feeds

	Slope	Slope CI	Intercept	Intercept CI	R^2	RMSE
In vitro gas production						
*b*GP	0.78	0.63 to 0.94	15.30	5.02 to 25.59	0.96	1.12
*c*GP	0.36	-0.08 to 0.81	6.02	2.38 to 9.65	0.40	0.30
GP_{24}	0.80	0.63 to 0.97	12.87	2.38 to 23.35	0.96	1.11
dOM	0.84	0.60 to 1.08	13.73	-7.03 to 34.49	0.92	0.91
ME	0.89	0.67 to 1.12	1.37	-1.67 to 4.40	0.94	0.08
$EDST_{IN\_SITU}$						
k = 5%/h	0.81	0.71 to 0.90	19.42	11.00 to 27.84	0.99	0.46
k = 8%/h	0.84	0.76 to 0.92	16.54	9.55 to 23.53	0.99	0.53
aCP_{IN_SITU}	0.92	0.54 to 1.29	15.42	-3.34 to 34.18	0.86	4.48
bCP_{IN_SITU}	0.95	0.58 to 1.32	-9.26	-28.31 to 9.80	0.87	4.37
cCP_{IN_SITU}	5.21	-0.40 to 10.83	-279.40	-992.30 to 433.40	0.46	468.50
$lagCP_{IN\_SITU}$	0.06	-2.19 to 2.31	0.24	-0.46 to 0.93	<0.01	0.36

*b*GP = potential gas production; *c*GP = rate of gas production; GP_{24} = corrected gas production at 24 h; dOM = digestibility of organic matter; ME = metabolisable energy; $EDST_{IN\_SITU}$ = effective protein degradation for ruminal passage rates of 5%/h and 8%/h; RMSE = root mean square error.

In Chapter 5.3.2. it was discussed that the heat used during pelleting in the present work was probably not enough to facilitate significant ST gelatinisation. However, feed processing may increase the microbial attachment due to particle size change, increasing the ruminal ST degradability (Huntington, 1997; Tothi *et al*., 2003). Goelema *et al*. (1998) found that ST degradability of pressure toasted mixtures of peas, lupins and faba beans was higher than calculated from single feeds, and that this difference was not related to the extent of ST gelatinisation. However, no ST-rich cereal grains were used in their study. In the present work, observed **$EDST_{IN\_SITU}$** was higher than calculated in all compound feeds, with a maximum of 4 pp for k = 8%/h. While the slopes and intercepts were significantly different from 1 and 0 respectively, due to small numerical differences between calculated and observed values, high R^2 value of 0.99, and small RMSE value, the differences between calculated and observed $EDST_{IN\_SITU}$ originated mainly from change of aST_{IN_SITU}. The aST_{IN_SITU} was higher for all pelleted compound feeds when compared with mash, from 4 up to 19 pp. While observed aST_{IN_SITU} could be relatively accurately predicted from single feeds (R^2 = 0.86), the RMSE value was considered to be high at 4.48. Further, the bST_{IN_SITU} was lower in all pelleted compound feeds when compared with mash, up to 19 pp. The calculation of cST_{IN_SITU} and $lagST_{IN\_SITU}$ from single feeds suffered from extreme values observed in wheat bran (Manuscript 1), resulting in low accuracy of prediction (R^2 = 0.46). However, it is known that the same feed processing procedure may affect the extent of ruminal degradability of ST differently (Mills *et al*., 1999; Ljøkjel *et al*., 2003). This could possibly lead to associative effects of cST_{IN_SITU}, yet this coud not be demonstrated in the present work. The possible associative effects of the degradation rate among single feeds when processed should be further researched using *in vitro* studies. Still, differences in $EDST_{IN\_SITU}$ were considered of low relevance for practical feed formulation. This was probably due to low impact of c_{IN_SITU} on ED_{IN_SITU} (Chapter 5.2.2.), and overall high ruminal ST degradation in all single and compound feeds. It was concluded that the ruminal ST degradation of single feeds in pelleted compound feeds can be considered additive.

In this chapter, it was shown that the additivity of single feeds in pelleted compound feeds was given for GP_{24}, dOM, ME, and $EDST_{IN\_SITU}$. However, in the present thesis only one pelleting procedure was examined, and it didn't involve high temperature that could facilitate big structural changes in protein and ST. Future research should encompass pelleting procedures with different production parameters (mainly heat and addition of moisture), and also other commonly used processing procedures like expanding and pressure toasting, to further describe potential associative effects occurring during commonly used feed processing procedures on ruminal degradation of nutrients and feeding values of compound feeds.

5.5. Evaluation of ruminal $InsP_6$ degradation in selected single and compound feeds

Phosphorus can be found in plant seeds and grains, most commonly bound in phytate. Phytate is any salt of phytic acid ($InsP_6$). For the release of bound P that can be absorbed by animals, the enzyme phytase is required. Unlike monogastric animals, ruminants can hydrolyse $InsP_6$ easily due to very high phytase activity of rumen microbiota (Raun *et al.*, 1956). Therefore, the main site of $InsP_6$ degradation in cows is the rumen (Ray *et al.*, 2013). The extent of ruminal $InsP_6$ degradation, while generally high, is known to vary among single feeds (Bravo *et al.*, 2000). Publications on effective ruminal degradability of $InsP_6$ (ED_{InsP6}) of single feeds and feed mixtures for ruminants are scarce, and no previous research on additivity of $InsP_6$ degradation in ruminants is known to the author. Because compound feeds are commonly fed in pelleted form, the importance of feed processing on ruminal $InsP_6$ of feed mixtures is important. High heat treatment (133°C and higher) has been shown to decrease the extent of ruminal $InsP_6$ degradation of single feeds shifting the site of digestion to the intestine, but once there the InsP is considered mostly unavailable for the animal (Konishi *et al.*, 1999; Park *et al.*, 2000). It is however not known if heat produced during pelleting (up to 80–90°C in the present work) is high enough to affect the ruminal $InsP_6$ degradation of compound feeds. Thus, in the present thesis the $InsP_6$ concentrations and ED_{InsP6} of single feeds and compound feeds in mash and pelleted form were determined. It was tested if $InsP_6$ concentrations and ED_{InsP6} of compound feeds can be calculated from single feeds, and if pelleting has a significant influence on ED_{InsP6} of compound feeds. Two compound feeds (4 and 5) were chosen in mash and pelleted form with all single feeds contained therein (maize, wheat, barley, soybeans, soybean meal, rapeseed meal, sunflower meal, faba beans, and DDGS). Ruminal degradation of $InsP_6$ was evaluated using the *in situ* procedure (Manuscript 3).

The **$InsP_6$ concentrations** among single feeds varied widely. The $InsP_6$ concentration was highest in oilseed meals: sunflower meal (32.9 g/kg DM), rapeseed meal (24.1 g/kg DM), and soybean meal (17.0 g/kg DM), followed by legume seeds: soybeans (14.4 g/kg DM) and faba beans (14.3 g/kg DM), cereal grains: wheat (8.2 g/kg DM), maize (7.0 g/kg DM), and barley (6.3 g/kg DM), and was smallest in DDGS (4.6 g/kg DM). The $InsP_6$ concentrations of compound feeds were calculated from $InsP_6$ concentrations of single feeds. While calculated and observed $InsP_6$ concentrations were similar for compound feed 5, for compound feed 4 the difference was considered to be large (Table 6). The observed $InsP_6$ concentration of compound feed 4 was higher than calculated by 2.1 g/kg DM. When including the sum of all $InsP_5$ isomers, the

additivity of InsP concentration was also not given in compound feed 4. The reason for this is not known.

Table 6. Calculated and observed concentrations of inositol phosphates ($InsP_6$ and sum of $InsP_5$ isomers) of compound feeds (g/kg DM)

Compound feed		$InsP_6$	∑ of $InsP_5$	$InsP_6$ + ∑ of $InsP_5$
4	Calculated	6.6	0.9	7.5
	Mash	8.7	1.2	9.9
	Pellet	8.9	0.9	9.8
5	Calculated	14.5	1.6	16.2
	Mash	14.4	1.7	16.1
	Pellet	12.6	1.5	14.1

Pelleting did not have a large effect on $InsP_6$ concentration in compound feed 4, but it lowered the $InsP_6$ concentration of compound feed 5 by 1.8 g/kg DM (Table 6). Plant intrinsic phytase activity differs among feedstuffs and even among genotypes within the same species (Rodehutscord *et al.*, 2016). The relevance of intrinsic phytase for $InsP_6$ hydrolysis in the rumen is not well understood, but is presumed to be small. However, the significance of intrinsic phytase may be of relevance for the present work. Milling can induce the activity of intrinsic plant phytase, resulting in $InsP_6$ hydrolysis and increased $InsP_5$ share (Lehrfeld, 1989; Kasim and Edwards, 1998). All feed samples in the present study were pre-ground in the feed mill (3 mm sieve size), and also ground for the *in situ* procedure (2 mm sieve size), with pelleted compound feeds going through additional grinding during the processing procedure. This could cause the activation of intrinsic phytase from single feeds in mixtures, leading to decrease in $InsP_6$ concentration in pelleted compound feeds. However, the sum of $InsP_5$ isomers was also lower in pelleted compound feeds when compared to mash, and no lower InsPs were found. Even when accounting for the sum of $InsP_5$ isomers together with the $InsP_6$, the difference remains high (2.0 g/kg DM), and therefore the activity of intrinsic phytase was likely not the reason for this difference. The $InsP_6$ can be bound in the phytate-protein complex in some single feeds (Selle *et al.*, 2000; Kies *et al.*, 2006). In the present study, the CP concentration was smaller in pelleted feeds when compared to mash, but only by 0.9 and 0.1 pp of CP in DM in compound feeds 4 and 5, respectively. No conclusion was possible because of the limited number of tested samples in the present work.

The **ED_{InsP6} values** also varied widely among single feeds. The ED_{InsP6} for k = 8%/h was highest in faba beans (91%), maize (90%), and DDGS (89%), and lowest in rapeseed meal (48%). The results of ED_{InsP6} degradation of most single feeds was high, and

reinforces previous knowledge of a good potential for $InsP_6$ hydrolysis in ruminants. However, this was not the case for rapeseed meal sample used in the present work. In rapeseed meal, $InsP_6$ is bound to protein in phytate-protein complexes (Serraino and Thompson, 1984), and thus the degradation of CP in rapeseed meal should be related to degradation of $InsP_6$. Heat treatment of rapeseed meal is known to affect its ruminal CP degradability (Nia and Ingalls, 1992). In Manuscript 1, the $ED_8CP_{IN\_SITU}$ of rapeseed meal was one of the lowest among single feeds, and amounted to 63%. Such small ED_{InsP6} value is therefore probably related to small $EDCP_{IN\_SITU}$. Comparison of disappearance of CP and $InsP_6$ from bags is presented in Figures 26–30.

In the present work, no associative effects of ruminal $InsP_6$ degradation among single feeds were observed in mash compound feeds. Calculated and observed values of ED_{InsP6} of compound feeds were similar. In compound feed 4 the ED_{InsP6} was overestimated by 1 pp, and in compound feed 5 the ED_{InsP6} was underestimated by 3 pp for both k = 5 and 8%/h, which was considered to be negligible, and additivity of ED_{InsP6} values from single feeds to mash compound feeds was considered to be given.

To test if pelleting affects the ruminal degradation of $InsP_6$ in compound feeds, effect of pelleting was evaluated in the present thesis by comparing $InsP_6$ degradation of mash and pelleted compound feeds. Pelleting of compound feeds in the present study increased ED_{InsP6} for 7 and 8 pp in compound feed 4 and 5 for k = 8%/h, respectively. The cause of such large increase of ED_{InsP6} after pelleting is probably methodologically coupled with increased disappearance of CP and ST due to particle size decrease in pelleted compound feeds, as discussed in Chapter 5.3.1. This was evident by the decrease of *a* fraction in pelleted compound feeds by 15 and 18 pp in compound feeds 4 and 5, respectively. This issue may lead to large overestimation of ruminal $InsP_6$ degradation. It is possible that smaller bag pore size than the current size of 50 µm could be used in future studies to alleviate feed particle loss. Pore sizes as low as 40 µm are within the commonly recommended range for *in situ* studies (Nocek, 1988; Vanzant *et al*., 1998). The common *in vitro* rumen simulation techniques also rely on incubation of feed samples in bags (Czerkawski and Breckenridge, 1977), and therefore may suffer from similar issues regarding feed particle loss. Even if feed samples would be incubated in a closed batch culture without the usage of bags, the difference in particle size between mash and pelleted feeds would presumably affect the rate of degradation of nutrients due to increased surface area for microbial attachment.

While the additivity of ED_{InsP6} from single feeds was given in mash compound feeds, additivity of ED_{InsP6} degradation of single feeds was not given for pelleted compound feeds. Associative effects in pelleted compound feeds were high, with ED_{InsP6} underestimation for k = 8%/h of 6 and 11 pp in compound feeds 4 and 5, respectively.

Such associative effects may be related to particle size change as a result of pelleting as described in Chapter 5.3.1., and intrinsic phytase activity of single feeds that interacted in compound feeds, inducing the $InsP_6$ degradation even before ruminal incubation. Associative effects were numerically much higher in pelleted compound feed 5 when compared to compound feed 4. Because the particle size distribution of those two compound feeds was similar (Manuscript 1), it can be presumed that the difference in the magnitude of associative effects was related to the intrinsic phytase activity. However, this was not measured in the present work. Major components in compound feed 4 were barley (46.5% in DM), soybeans (17.8% in DM), and faba beans (16.1% in DM), and in compound feed 5 maize (31.6% in DM), rapeseed meal (17.3% in DM), and faba beans (16.3% in DM). Since maize and soybeans typically do not possess a high phytase activity, barley in compound feed 4 probably provided the majority of intrinsic phytase activity (Eeckhout and De Paepe, 1994). This is however contrary to the observed results, where compound feed 5 had a higher decrease in ED_{InsP6} as a result of pelleting. Likewise, the possible effect of intrinsic phytase activity on $InsP_6$ concentrations of compound feeds was previously rejected in this chapter. Further research is necessary, since only two compound feeds were examined in the present work, however potential combination of particle size decrease and intrinsic phytase activity may play a role in ruminal degradation of $InsP_6$ from pelleted compound feeds *in vivo*. The retention time of feed in the rumen of high producing dairy cows is relatively low, and this may limit the microbial attachment and potential for microbial phytate degradation (Jarrett *et al*., 2014). Therein, the lowering of feed particle size and the initial $InsP_6$ degradation by intrinsic phytase in pelleted compound feeds may enable greater utilisation of feed P.

In seeds of most cereal grains and legumes the $InsP_6$ is stored in globoids, within protein storage vacuoles, and in legumes it was previously found to bind proteins in protein-phytate complexes (Urbano *et al*., 2000; Selle *et al*., 2012). Because of the existence of such protein-phytate complexes, feed processing that affects the degradability of protein may presumably affect the extent of $InsP_6$ hydrolysis. Therefore, degradation of $InsP_6$ and CP may be related in samples of legumes. **Ruminal disappearance of $InsP_6$ and CP** was found to correlate well in rapeseed meal and to a lesser extent in soybean meal (Haese *et al*., 2017). However, for cereal grains the correlations were not as good, presumably due to globoids containing $InsP_6$ not being placed in protein storage vacuoles in cereals (O'Dell *et al*., 1972). In maize, $InsP_6$ is storage mostly in the germ (O'Dell *et al*., 1972). In other cereals, however, $InsP_6$ is stored in globoids found in the aleurone layer (O'Dell *et al*., 1972; Tanaka *et al*., 1974). In most oilseeds and legumes, globoids are found in the kernel (Viveros *et al*., 2000). This variety of $InsP_6$ localisation and especially the existence of protein-phytate

complexes indicates that different single feeds may have different extent of correspondence between CP and $InsP_6$ degradation.

In the present work linear regressions indicated low agreement between ruminal disappearance of $InsP_6$ and CP in cereals (R^2 = 0.77, 0.84, and 0.56 in maize, wheat, and barley, respectively; Figure 26). The ruminal disappearance of $InsP_6$ and CP was in good agreement in DDGS (R^2 = 0.93; Figure 26), soybeans (R^2 = 0.97; Figure 27) and all oilseed meals (R^2 = 0.96, 0.96, and 0.97 in soybean meal, sunflower meal, and rapeseed meal, respectively; Figure 28), but not for faba beans (R^2 = 0.87; Figure 27). The results for faba beans were surprising, because phytate in faba beans is known to form complexes with proteins (Rosa-Sibakov *et al.*, 2018), just like other legumes. The $InsP_6$ concentration in DDGS was low (4.6 g/kg DM), and a_{IN_SITU} of $InsP_6$ (77%) and ED_{InsP6} were high (89% for k = 8%/h). Also, the aCP_{IN_SITU} of DDGS was high (53%). Hence, it may be that the good correspondence of $InsP_6$ and CP disappearance is misleading because of rapid disappearance of $InsP_6$ and CP from bags. The smallest correspondence of $InsP_6$ and CP disappearance of single feeds was seen for barley. While barley is a cereal, and thus lacks phytate-protein complexes, such small correspondence was not expected. This was probably caused by an artefact regarding *in situ* $InsP_6$ disappearance data, where the mean 0-hour $InsP_6$ loss amounted to 58%, but the mean 2-, 4-, and 6-h losses amounted to 42, 48, and 77%, respectively. The reason for 2-h incubation loss being higher than 0-h incubation loss is not known. The mean loss of CP was 34% at 0- and 68% at 2-h incubation time point.

Finally, it was concluded that $InsP_6$ disappearance can be reliably predicted from CP disappearance for samples of soybeans, soybean meal, sunflower meal, and rapeseed meal.

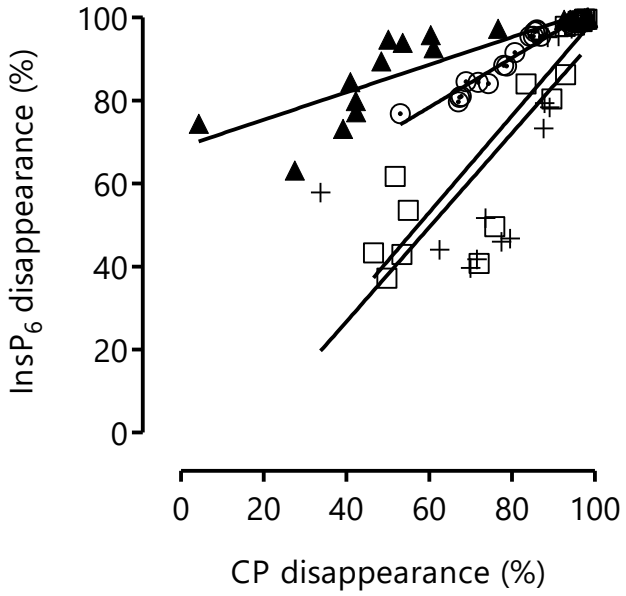

Figure 26. Linear regressions of CP and $InsP_6$ disappearance of cereal grains and DDGS. Maize (▲): y = 0.33x + 68.72, R^2 = 0.77, RMSE = 5.48; Wheat (□): y = 1.17x - 16.92, R^2 = 0.84, RMSE = 10.36; Barley (+): y = 1.14x - 18.66, R^2 = 0.56, RMSE = 16.58; Dried distillers' grains with solubles (DDGS) (ʘ): y = 0.60x + 42.62, R^2 = 0.93, RMSE = 2.06.

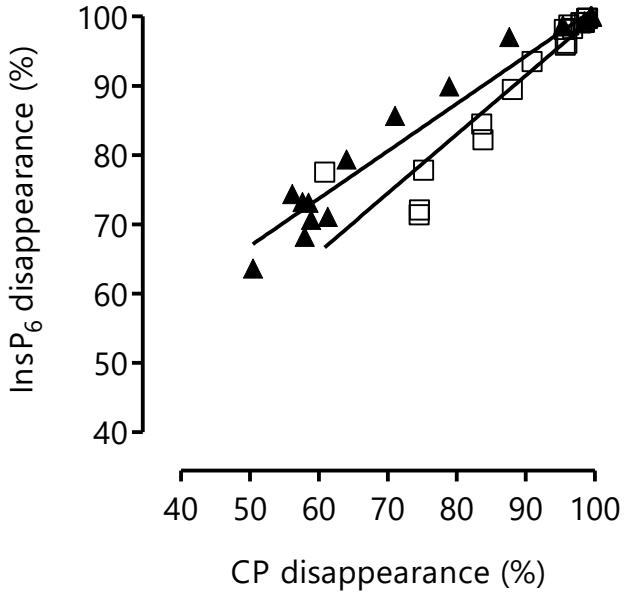

Figure 27. Linear regressions of CP and $InsP_6$ disappearance of legume seeds. Soybeans (▲): y = 0.69x + 32.59, R^2 = 0.97, RMSE = 2.48; Faba beans (□): y = 0.85x + 14.98, R^2 = 0.87, RMSE = 3.75.

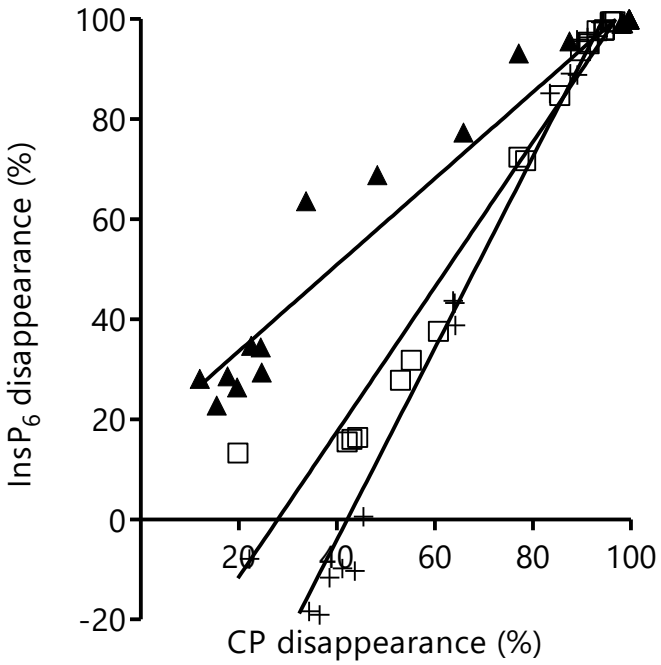

Figure 28. Linear regressions of CP and $InsP_6$ disappearance of oilseed meals. Soybean meal (▲): y = 0.86x + 16.33, R^2 = 0.96, RMSE = 6.56; Sunflower meal (□): y =1.45x - 40.54, R^2 = 0.96, RMSE = 7.79; Rapeseed meal (+): y = 1.92x - 80.74, R^2 = 0.97, RMSE = 8.36.

Different results for single feeds depending on the feed group indicate that the possibility of estimation of $InsP_6$ ruminal disappearance from CP disappearance in compound feeds will depend on their single feed composition. Prediction of $InsP_6$ disappearance from CP disappearance was not accurate in compound feed 4 (R^2 = 0.84 and 0.86 for mash and pellet, respectively, Figure 29). Compound feed 4 contained 56.1% cereal grains (9.6% maize and 46.5% barley), and 16.1% faba beans vs. 17.8% soybeans, 4.9% soybean meal and 5.1% DDGS in DM. On the contrary, compound feed 5 contained 43.3% cereal grains (31.6% maize and 11.7% wheat) and 16.3% faba beans, vs. 35.2% oilseed meals (7.9 % soybean meal, 17.3% rapeseed meal, and 10.0% sunflower meal) and 5.2% DDGS in DM. In compound feed 5, prediction of $InsP_6$ disappearance from CP disappearance was more accurate (R^2 = 0.93 and 0.94 for mash and pellet, respectively, Figure 30). Accuracy of prediction was similar between mash and pelleted form in both compound feeds. Finally, it was concluded that accuracy of prediction of $InsP_6$ disappearance from CP disappearance in compound feeds depends on single feeds composition, particularly on the ratio of cereal grains and oilseed meals.

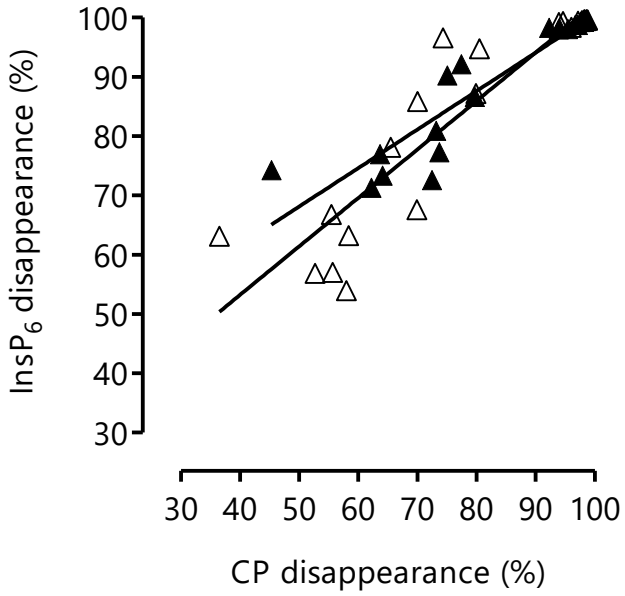

Figure 29. Linear regression of CP and $InsP_6$ disappearance of compound feed 4 in mash and pelleted form. Mash (△): y = 0.82x + 20.53, R^2 = 0.84, RMSE = 7.13; Pellet (▲): y = 0.65x + 35.67, R^2 = 0.86, RMSE = 4.30.

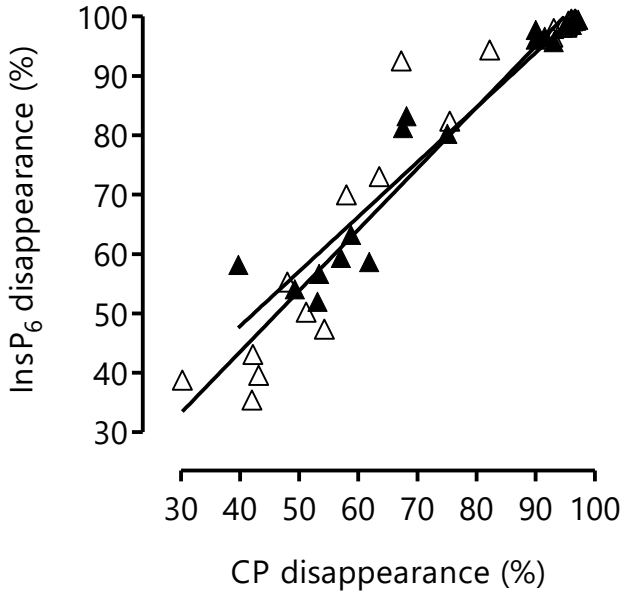

Figure 30. Linear regression of CP and $InsP_6$ disappearance of compound feed 5 in mash and pelleted form. Mash (△): y = 1.03x + 2.35, R^2 = 0.93, RMSE = 6.95; Pellet (▲): y = 0.92x + 11.02, R^2 = 0.94, RMSE = 4.90.

In this chapter, ruminal degradation of InsP from single and compound feeds was characterised. Additivity of ruminal $InsP_6$ degradation from single feeds in compound feeds in mash form was given, but not for compound feeds in pelleted form. Pelleting increased ruminal $InsP_6$ degradation, probably only due to methodological reasons (change in feed particle size, and increased washout of feed particles through the bag pores). Ruminal disappearance of $InsP_6$ could be predicted from disappearance of CP in samples of soybeans, oilseed meals, but not in DDGS, faba beans and cereal grains. This difference in single feeds was reflected in two examined compound feeds depending on the single feed composition. As only two compound feeds were used to evaluate additivity and effects of pelleting on ED_{InsP6} in this thesis, results should be interpreted with caution. Further studies are recommended, using a higher number of compound feeds with a wide range of single feeds.

5.6. Prediction of ruminal CP degradation of compound feeds

Two approaches for prediction of ruminal CP degradation of compound feeds were tested. Firstly, prediction of ruminal CP degradation from CP fractions of CNCPS model and proximate nutrients was attempted. Then, prediction of ruminal CP degradation of compound feeds from ruminal DM degradation of either single feeds (calculated values) or compound feeds (observed values) was attempted.

5.6.1. Prediction of ruminal CP degradation of compound feeds from CP fractions

Determination of feeding value of feedstuff for ruminants often involves fistulated animals, either for *in situ* trials, or alternatively as a donor of rumen fluid for different *in vitro* techniques. To eliminate the need for fistulation of animals, different laboratory methods were developed to chemically separate CP and carbohydrate fractions of feedstuffs into fractions with variable rates of degradation in the rumen. One method is the CNCPS (Sniffen *et al*., 1992), which was used in the present thesis for fractionation of CP. Five chemically determined CP fractions have distinctly different assumed rates of ruminal degradation. Fraction A is considered instantly soluble in the rumen, fractions B1, B2, and B3 are potentially degradable in the rumen, and fraction C is considered undegradable in the rumen and passing to the small intestine. Using determined CP fractions, table values of ruminal degradation rates for each specific feed and ruminal passage rates, ruminal CP degradation values of feeds can be calculated. The possibility for **prediction of EDCP** was discussed in Manuscript 2. The EDCP values estimated using CNCPS (ED_{CNCPS}) were compared with EDCP values determined *in situ* (ED_{IN_SITU}) for k = 5 and 8%/h. The ED_{CNCPS} were calculated using equations of both Shannak *et al.* (2000) and Fox *et al.* (2003), with only the latter shown in Manuscript 2. Prediction of EDCP of single feeds from CP fractions was, for both equations, not accurate (Table 7).

Table 7. Comparison of ruminal effective crude protein degradability values of single feeds estimated using CNCPS model (ED_{CNCPS}) and determined using the *in situ* procedure (ED_{IN_SITU}) for ruminal rate of passage of 8%/h

Single feed	ED_{CNCPS} [1]	ED_{CNCPS} [2]	ED_{IN_SITU} [3]
Maize	83	55	61
Wheat	108	73	81
Barley	89	69	82
Soybeans	93	45	75
Soybean meal	93	62	55
Rapeseed meal	45	65	63
Sunflower meal	67	69	72
Faba beans	92	75	89
DDGS[4]	-168	50	78
Maize gluten	76	75	87
Wheat bran	74	74	82
Sugar beet pulp	71	78	64

[1]ED_{CNCPS} calculated using equations by Shannak *et al.* (2000); [2]ED_{CNCPS} calculated using equations by Fox *et al.* (2003); [3]ED_{IN_SITU} determined in Manuscript 1; [4]DDGS = dried distillers' grains with solubles.

Shannak *et al.* (2000) evaluated degradation of a wide range of concentrate single feeds, and compound feeds made thereof. They found that EDCP can be accurately calculated from CP fractions. However, not all single feeds from the present thesis were contained in that data pool (maize, faba beans, DDGS, and wheat bran). The ED_{CNCPS} values of single feeds calculated using the equation of Shannak *et al.* (2000) corresponded reasonably well only with ED_{IN_SITU} values of sunflower meal (67 vs. 72%) and faba beans (92 vs. 89%), and had big deviations for other single feeds. When using equations of Fox *et al.* (2003) for calculation of ED_{CNCPS} (Manuscript 2), good correspondence between ED_{CNCPS} and ED_{IN_SITU} has been seen only for rapeseed meal (65 vs. 63%) and sunflower meal (69 vs. 72%). Overall, prediction of ED_{IN_SITU} of single concentrate feeds using CP fractions was considered to be unsuccessful in the present work.

For compound feeds, the ED_{CNCPS} values from equations of Shannak *et al.* (2000) in most cases underestimated or overestimated ED_{IN_SITU} with large differences (Table 8).

Table 8. Comparison of ruminal effective crude protein degradability values of compound feeds estimated using CNCPS model (ED_{CNCPS}) and determined using the *in situ* procedure (ED_{IN_SITU}) for ruminal rate of passage of 8%/h

Compound feed		ED_{CNCPS} [1]	ED_{CNCPS} [2]	ED_{IN_SITU} [3]
1	mash	81	67	77
	pellet	79	66	77
2	mash	65	69	81
	pellet	66	69	83
3	mash	82	70	88
	pellet	79	69	86
4	mash	86	62	79
	pellet	85	63	85
5	mash	77	66	74
	pellet	80	65	79
6	mash	72	66	80
	pellet	78	64	83
7	mash	61	65	78
	pellet	66	64	80
8	mash	75	65	79
	pellet	77	64	81

[1]ED_{CNCPS} calculated using equations by Shannak *et al.* (2000); [2]ED_{CNCPS} calculated using equations by Fox *et al.* (2003); [3]ED_{IN_SITU} determined in Manuscript 1.

Acceptable correspondence between ED_{CNCPS} and ED_{IN_SITU} values was found for mash compound feeds 1 (81 vs. 77%), 5 (77 vs. 74%), and 8 (75 vs. 79%), and for pelleted compound feeds 1 (79 vs. 77%), 4 (85 vs. 85%), 5 (80 vs. 79%), 6 (78 vs. 83%), and 8 (77 vs. 81%). It is not clear why the prediction of ED_{IN_SITU} from ED_{CNCPS} was more accurate for some compound feeds than for the other. An attempt to predict ED_{IN_SITU} from ED_{CNCPS} that was calculated using the equation of Fox *et al.* (2003) was presented in Manuscript 2. The ED_{CNCPS} values from equations of Fox *et al.* (2003) underestimated ED_{IN_SITU} values in all compound feeds. Underestimation of ED_{IN_SITU} was between 11 and 22 pp for mash and between 8 and 18 pp in pelleted compound feeds. It was concluded that the accurate prediction of ED_{IN_SITU} of compound feeds using CP fractions is not possible. Concerning the calculation of ED_{CNCPS} using equations of Fox *et al.* (2003), it can be that ruminal degradation rates taken from a table did not correspond to the one in single feed samples in the present work. Furthermore, the

ruminal degradation rates of compound feeds were not found in literature, and were calculated for compound feeds from table values of ruminal degradation rates for single feeds. This could have been a possible source of error in calculation of ED_{CNCPS} values of compound feeds, leading to non-systematic deviations of ED_{CNCPS} from ED_{IN_SITU}, resulting in the low accuracy of prediction.

In the next step, development of equation to predict $EDCP_{IN\_SITU}$ of compound feeds was attempted. Stepwise linear multiple regression was performed using procedure REG (selection stepwise; version 9.4 of SAS system for Windows, SAS Institute, NC, USA), and significance level set on 0.1. Variables in the model included the proximate nutrients (Manuscript 1), and determined CP fractions (Manuscript 2), but no variable was significant for prediction of $EDCP_{IN\_SITU}$. Finally, it was concluded that prediction of ruminal CP degradation of compound feeds in the present thesis was not possible using CP fractions.

5.6.2. Prediction of ruminal CP degradation of compound feeds from ruminal DM degradation of single feeds or compound feeds

The ruminal degradation of DM is easier to determine than CP, as no chemical analysis is necessary. The ruminal DM degradation of feed samples in the present thesis is presented in Annexes 2c and 5. The $EDDM_{IN\_SITU}$ and $EDCP_{IN\_SITU}$ values were previously found to be related for some single feeds (Ha and Kennelly, 1984). If $EDCP_{IN\_SITU}$ of compound feeds could be predicted from $EDDM_{IN\_SITU}$ of single feeds, this would potentially enable rapid estimation of $EDCP_{IN\_SITU}$ for any combination of single feeds. Thus, in the present thesis the $EDDM_{IN\_SITU}$ of compound feeds **calculated from single feeds** were compared to $EDCP_{IN\_SITU}$ values of compound feeds in mash form. Due to low accuracy of prediction (R^2) and high RMSE value (Figure 31), the prediction of $EDCP_{IN\_SITU}$ of compound feeds from calculated $EDDM_{IN\_SITU}$ was considered not reliable.

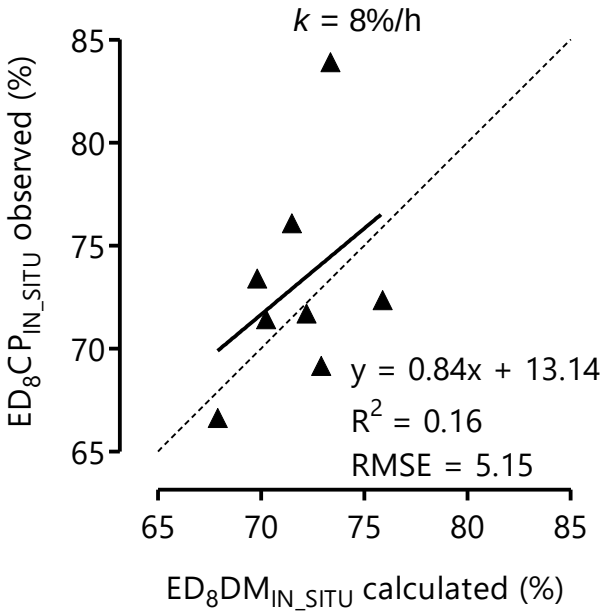

Figure 31. Prediction of $ED_8CP_{IN\_SITU}$ of compound feeds from $ED_8DM_{IN\_SITU}$ of compound feeds calculated from single feeds

Following the attempt of prediction of $ED_8CP_{IN\_SITU}$ of compound feeds from calculated $ED_8DM_{IN\_SITU}$ of compound feeds that was not successful, it was presumed that the prediction of $ED_8CP_{IN\_SITU}$ of compound feeds **from observed $ED_8DM_{IN\_SITU}$ of compound feeds** could be more accurate. This resulted in higher accuracy of prediction (R^2 = 0.34) and smaller RMSE value (4.56), yet it was still not considered to be reliable for practical purposes (Figure 32). In both approaches, the $ED_8CP_{IN\_SITU}$ was both underestimated and overestimated, depending on the compound feed. Presumably, ruminal CP and DM degradation were not related in all single feeds, and this was mirrored in compound feeds. It was concluded that the prediction of $ED_8CP_{IN\_SITU}$ of compound feeds from either calculated or observed $ED_8DM_{IN\_SITU}$ was not possible for samples in the present thesis.

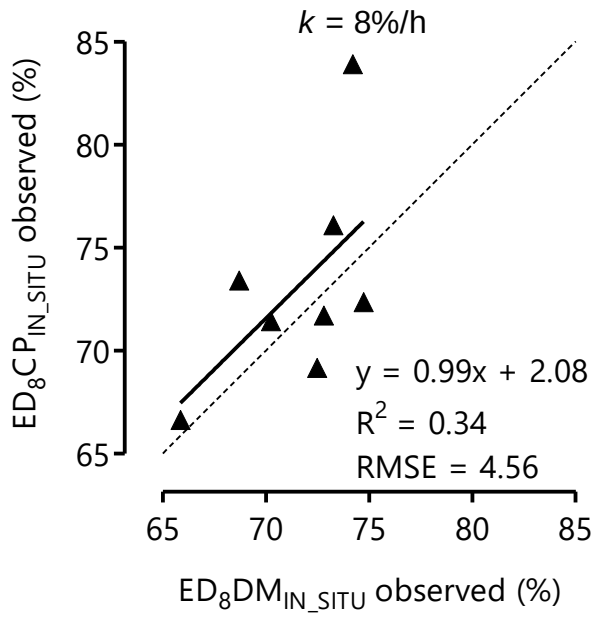

Figure 32. Prediction of $ED_8CP_{IN\_SITU}$ of compound feeds from $ED_8DM_{IN\_SITU}$ of compound feeds

It was concluded that ruminal DM degradation of single feeds or compound feeds cannot be used for prediction of ruminal CP degradation of compound feeds in the present thesis.

5.7. Overall conclusions and outlook

Because compound feeds are a major source of protein and energy in dairy cow ration, associative effects among single feeds in compound feeds can have a major influence on energy and protein supply. In the present thesis, the additivity of single feeds to mash compound feeds was considered to be given for *in situ* determined ED of CP, ST, and $InsP_6$. However, individual degradation parameters (*a, b, c*, and lag) were not always additive. Current feeding models usually rely only on ED values of feedstuff, but it is possible that future models will also include individual degradation parameters.

Some associative effects during *in situ* studies may be related to physical properties of single feeds. In Manuscript 1 the potential effect of viscosity on loss of particles from bags was discussed. However, to test this hypothesis more research on interactions of physical characteristics of single feeds inside *in situ* bags is needed, using viscous single feeds like maize gluten, and feeds that have a tendency of clumping together, like barley (Vanzant *et al*., 1998).

Additivity of single feeds in compound feeds was considered to be given for the *in vitro* estimated GP_{24}, dOM, uCP, and CP fractions. However, large associative effects were found for ID_{RUP}. The lack of additivity of ID_{RUP} is remarkable, but it may be related to methodological causes. Validation of ID_{RUP} results with the mobile bag technique is recommended in future additivity studies. The associative effects could not be related to any specific single feed, because not all single feeds were present in all compound feeds.

Additivity of ME depended on the equations which are used for calculation of ME from measured GP_{24}. Current ME equations are usually specific for a particular feed type, but the same equations across all single and compound feeds should be used for appropriate evaluation of additivity. When the same ME equations for all single and compound feeds were used (GfE, 2009), the additivity of ME values was given.

Pelleting had a relatively small effect on the ruminal degradation of nutrients and feeding values of compound feeds, except for the ID_{RUP}. This is presumably a result of temperature during the pelleting process not being excessively high (up to 80–90°C), thus not resulting in large change of nutritional value of compound feeds. Pelleting increased the share of fine feed particles in all compound feeds, and during the *in situ* study the loss of undegraded particles through bag pores presumably occurred.

Additivity of single feeds in pelleted compound feeds was also evaluated. It was concluded that the additivity of *in situ* determined ED of CP and ST, and *in vitro* determined CP fractions, GP_{24}, dOM, and ME of single feeds in pelleted compound feeds was given. However, large associative effects were found for *in situ* determined

ED of $InsP_6$ and *in vitro* determined uCP and ID_{RUP}. Only one pelleting procedure was used in the present thesis, and further research on additivity of feeding values of single feeds in compound feeds is suggested using different feed processing procedures, and variable heat and moisture levels.

While the present thesis attempted to utilise some of the most commonly used single concentrate feeds in Germany, perhaps the results could be different if other single feeds were chosen. This should be explored in further studies. Following further expanding of the data base on associative effects among single feeds, artificial neural networks could be utilised to predict feed interactions. This would necessitate applying the standardised method across the trials, and using a wide variety of single feeds in fixed steps of inclusion levels. The results from such approach could potentially lead to precise models for estimation of potential associative effects from any combination of single feeds and their respective inclusion levels. However, compound feeds are only one part of the dairy cow ration. Development of models that consider all feed interactions relevant for diets of high-yielding dairy cows must also include interactions between forages and concentrates when fed TMR.

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6. SUMMARY

The environmental impact of intensive animal farming has been steadily increasing. Cattle can contribute to environmental pollution due to relatively low nitrogen (**N**) and phosphorus (**P**) utilisation, leading to their excess excretion. High-yielding dairy cows are commonly fed concentrate compound feed, in mash or pelleted form, to satisfy high protein and energy requirements. Main source of energy in concentrate compound feeds is starch (**ST**). For the accurate formulation of compound feeds, comprehensive insight into nutritive values of single feeds as well as their potential interactions (associative effects) when mixed is needed. Typically, the nutritive values of single feeds are considered to be additive, assuming that no associative effects exist. However, data supporting such assumption for concentrate feed are scarce. The present thesis had two aims: evaluation of additivity of ruminal degradation of nutrients and feeding values of single concentrate feeds in compound feeds, and evaluation of effects of pelleting on ruminal degradation of nutrients and feeding values of compound feeds.

Twelve single feeds were used to formulate eight compound feeds in different combinations, targeting crude protein (**CP**) concentrations from 16 to 30% in dry matter (**DM**). Compound feeds were prepared both, in mash and pellet form in a commercial feed mill using standard industrial conditions. Ruminal degradation of single and compound feeds was evaluated using *in situ* and different *in vitro* techniques.

The *in situ* incubations were conducted by incubating samples of all single and compound feeds in polyester bags for 2, 4, 6, 8, 16, 24, 48, and 72 hours in three ruminally fistulated dairy cows. Bag residues were analysed and the ruminal effective degradability ($\mathbf{ED_{IN\_SITU}}$) of CP and ST, was calculated for passage rates of 5 and 8%/h. The *in vitro* gas production (**GP**), digestibility of organic matter (**dOM**), metabolisable energy (**ME**), and utilisable CP at the duodenum (**uCP**) were evaluated using Hohenheim Gas Test and extended HGT. Intestinal digestibility ($\mathbf{ID_{RUP}}$) of ruminally undegraded protein (**RUP**) was determined using a three-step enzymatic method through incubation with pepsin and pancreatin. Chemical fractionation of CP was performed according to the Cornell Net Carbohydrate and Protein System (**CNCPS**). Wet sieving procedure was performed to determine particle size distribution of all feed samples. Assessment of additivity was performed by comparing the observed values of compound feeds with values for compound feeds calculated from single feeds.

It was concluded that additivity of single feeds in mash compound feeds was given for $EDCP_{IN\_SITU}$, $EDST_{IN\_SITU}$ (Manuscript 1), uCP, CP fractions, GP, and dOM (Manuscript 2). Here, associative effects among single feeds were considered to be small and should not affect formulation of concentrate compound feeds. The GP and proximate nutrients are necessary to estimate ME using appropriate equations, often specific for feed or feed type. The additivity of ME was given only when same ME equation for single and compound feeds was used. Additivity was not given for ID_{RUP} (Manuscript 2). The reasons for big differences between calculated and observed values of ID_{RUP} for compound feeds could not be related to a specific single feed and remained unknown. The three-step method for estimation of ID_{RUP} may not be accurate enough for evaluation of additivity of wide range of single feeds, presumably because of the low variation of sample types used for validation of the method. The additivity of ID_{RUP} should be evaluated using the mobile bag technique in future studies.

Pelleting had overall small effects on feeding values of compound feeds determined *in situ* and *in vitro* (Manuscripts 1 and 2). Presumably, the relatively low intensity of heating (up to 80–90°C) during the pelleting process was not sufficient to significantly affect nutritive value of compound feeds, with the exception of decreased ID_{RUP}. Pelleting increased ED of CP and ST of compound feeds. However, this was probably caused by an increase of small feed particles as a result of the pelleting procedure, leading to higher disappearance of undegraded particles from the bags. This presumption was reflected in results of *in vitro* studies where pelleting increased uCP concentration in most compound feeds. The effect of pelleting on CP fractions was not big.

Additivity of single feeds was also evaluated for pelleted compound feeds. Additivity was considered to be given for $EDCP_{IN\_SITU}$, $EDST_{IN\_SITU}$ (Manuscript 1), CP fractions, GP_{24}, dOM, and ME, but not for ID_{RUP} (Manuscript 2). Further comprehensive research on additivity in processed compound feeds is necessary, with utilisation of variable processing conditions, particularly regarding heat and moisture.

Future animal nutrition research will focus on establishing more precise feeding models and lowering the usage of animals in trials. Estimation of feeding value of diets using only *in vitro* chemical analysis has a great potential, and one of the mathematical models for estimation of CP degradability is CNCPS. However, in the present thesis the CNCPS model could not accurately predict $EDCP_{IN\_SITU}$ (Manuscript 2). Further development of these systems is therefore recommended.

Phosphorus is located in plants as phytate (**$InsP_6$**). Ruminants are able to release P from $InsP_6$ more easily than non-ruminants, however ruminal $InsP_6$ degradation differs among single feeds. Two compound feeds and related single feeds were chosen to

characterise $InsP_6$ concentrations and ED_{InsP6}, and to evaluate additivity and effects of pelleting (Manuscript 3). Additivity was given for ED_{InsP6} of single feeds in mash compound feeds, but not in pelleted compound feeds. Pelleting significantly increased ED_{InsP6} of compound feeds, although the effect was probably related to the decrease in particle size and higher loss of undegraded particles through *in situ* bags.

Overall, it was concluded that additivity of ruminal degradation of nutrients and feeding values of single feeds in mash and pelleted compound feeds can be assumed for practical feed formulation. While some associative effects were detected, they might be related to methodological causes in most of the cases.

7. ZUSAMMENFASSUNG

Die Umweltauswirkungen der intensiven Tierhaltung nehmen stetig zu. Bei Rindern wird ein relativ großer Teil des durch das Futter zugeführten Stickstoffs (**N**) und Phosphors **(P)** wieder ausgeschieden. Um den hohen Protein- und Energiebedarf hochleistender Milchkühe zu decken werden diese in der Regel zusätzlich zum Grobfutter mit Kraftfuttermitteln, in Mehl- oder Pelletform, gefüttert. Die Hauptenergiequelle in Kraftfuttermitteln ist Stärke (**ST**). Die Synchronität des Rohprotein (**XP**)- und ST-Abbaus im Pansen ist von besonderer Bedeutung um eine höchstmögliche mikrobielle Proteinsynthese im Pansen zu erreichen und die Leistung sowie die Tiergesundheit zu erhalten. Für die Formulierung von Mischfuttermitteln sind die Kenntnis der Nährwerte der einzelnen Futtermittel sowie Wissen über mögliche Wechselwirkungen (assoziative Effekte) zwischen den Einzelkomponenten erforderlich. Typischerweise werden die Nährwerte einzelner Futtermittel als additiv betrachtet, was bedingt, dass assoziative Effekte ausgeschlossen werden. Forschungsdaten, die eine solche Annahme für Kraftfutter unterstützen sind jedoch unzureichend. Die vorliegende Arbeit hatte zwei Ziele: Die Bewertung der Additivität des ruminalen Nährstoffabbaus und der Futterwerte von Einzelfuttermitteln in Mischfuttermitteln, und die Bewertung der Auswirkungen einer Pelletierung auf den ruminalen Nährstoffabbau und die Futterwerte von Mischfutter.

Basierend auf zwölf gängigen Einzelfuttermitteln wurden acht Mischfuttermittel mit variablen Anteilen der Komponenten erstellt. Die Mischfutter wurden so konzipiert, dass XP-Konzentrationen von 16 bis 30% in der Trockenmasse (**TM**) erreicht wurden. Die Mischfutter wurden sowohl in Mehl- als auch in Pelletform in einem kommerziellen Mischfutterwerk unter industriellen Bedingungen hergestellt. Proben der Einzelfuttermittel und der mehlförmigen sowie pelletierten Mischfutter wurden *in situ*- und mit verschiedenen *in vitro*-Methoden hinsichtlich ihres Futterwerts untersucht.

Hierzu wurden die für 2, 4, 6, 8, 16, 24, 48 und 72 Stunden in drei Pansen fistulierten Milchkühen inkubiert. Die Beutelrückstände wurden chemisch analysiert und der effektive XP- und ST-Abbau ($\mathbf{ED_{IN\_SITU}}$) für eine Passagerate von 5 und 8%/h berechnet. Die *in vitro* Gasbildung (**GB**), die Verdaulichkeit der organischen Masse (**dOM**), umsetzbare Energie (**ME**) und das nutzbare XP am Duodenum (**nXP**) wurden im Hohenheimer Futterwerttest bzw. im erweiterten HFT untersucht. Die Dünndarmverdaulichkeit ($\mathbf{ID_{RUP}}$) des im Pansen nicht abgebauten Proteins (**RUP**) wurde in einem dreistufigen *in vitro* System basierend auf einer Inkubation mit Pepsin und Pankreatin bestimmt. Zudem wurde die chemische XP-Fraktionierung nach dem Cornell Net Carbohydrate and Protein System (**CNCPS**) durchgeführt. Die Partikelgrößenverteilung aller Proben wurde durch Nasssiebung bestimmt.

Die Ermittlung der Additivität erfolgte durch den Vergleich der ermittelten Werte für Mischfutter mit kalkulierten Werten für Mischfutter, die aus einzelnen Futtermitteln berechnet wurden.

Die Ergebnisse führten zu dem Schluss, dass die Additivität von Einzelfuttermitteln in mehlförmigen Mischfutter für $EDXP_{IN\_SITU}$, $EDST_{IN\_SITU}$ (Manuskript 1), nXP, CNCPS-Fraktionen, GP und dOM (Manuskript 2) gegeben war. Es wurde geschlussfolgert, dass assoziative Effekte zwischen den einzelnen Futtermitteln bei der Formulierung von Mischfutter nicht berücksichtigt werden brauchen. Die ME konnte mit ausreichender Schätzgüte aus der GB und den Rohnährstoffen abgeleitet werden. Diese Schätzung war in vielen Fällen spezifisch für den Futtertyp oder das Futter. Die Additivität von ME war nur gegeben, wenn die gleiche ME-Formel für Einzel- und Mischfutter verwendet wurde. Die Werte für ID_{RUP} zeigten jedoch keine Additivität (Manuskript 2). Es konnte kein Zusammenhang zwischen den im Mischfutter enthaltenen Einzelfuttermitteln und den assoziativen Effekten festgestellt werden. Es wurde daher geschlussfolgert, dass die dreistufige Methode zur Schätzung von ID_{RUP} möglicherweise nicht zur Bewertung der Additivität eines breiten Spektrums von Einzelfuttermitteln geeignet ist, vermutlich wegen der geringen Variation der Probentypen, die zur Validierung der Methode verwendet wurden. Die Additivität von ID_{RUP} sollte in zukünftigen Studien mit Hilfe der mobilen Beuteltechnik evaluiert werden.

Pelletieren hatte insgesamt einen sehr geringen Einfluss auf die *in situ* und *in vitro* ermittelten Kennzahlen des Futterwerts der hier untersuchten Mischfutter (Manuskript 1 und 2). Vermutlich war die relativ geringe Ausmaß der Erhitzung während des Pelletiervorgangs (80–90°C) nicht ausreichend, um den Nährwert von Mischfuttermitteln signifikant zu beeinflussen, mit der Ausnahme von reduziertem ID_{RUP}. Lediglich für ID_{RUP} konnte eine Veränderung nachgewiesen werden. Pelletieren hat die ED von XP und ST erhöht. Dies könnte an einer Verminderung der Partikelgröße durch das Pelletieren liegen, wodurch vermutlich mehr kleine Partikel aus dem Beutel ausgewaschen wurden. Diese Annahme spiegelte sich in den Ergebnissen von *in vitro*-Studien wider, bei denen Pelletieren die nXP-Konzentration in den meisten Mischfuttern erhöhte. Der Einfluss des Pelletierens auf die CNCPS-Fraktionen war gering.

Die Additivität der Werte für Einzelfuttermittel wurde auch für pelletierte Mischfuttermittel bewertet. Die Additivität war für $EDXP_{IN\_SITU}$, $EDST_{IN\_SITU}$ (Manuskript 1), CNCPS-Fraktionen, GP, dOM und ME gegeben, nicht aber für ID_{RUP} (Manuskript 2). Weitere umfassende Untersuchungen zur Additivität in prozessierten Mischfuttermitteln sind erforderlich. Hierbei sollten variable Verarbeitungsbedingungen, insbesondere hinsichtlich Wärme und Feuchtigkeit, untersucht werden.

Die zukünftige Forschung im Bereich der Tierernährung wird sich auf die Entwicklung präziserer Fütterungsmodelle und die Verringerung des Einsatzes von Tieren in Versuchen einstellen müssen. Die Schätzung des Futterwerts anhand von chemischen Analysen, z.B. mittels des CNCPS, birgt hierbei ein großes Potenzial. In der vorliegenden Arbeit konnte die EDXP allerdings nicht durch das CNCPS-Modell geschätzt werden (Manuskript 2). Eine Weiterentwicklung dieser Systeme wird daher empfohlen.

Phosphor liegt in Pflanzensamen größtenteils als Phytat (**$InsP_6$**) vor. Wiederkäuer sind durch die Vormagenfermentation in der Lage, P aus $InsP_6$ leichter freizusetzen als Nichtwiederkäuer. Der ruminale Abbau von $InsP_6$ ist jedoch zwischen Futtermitteln verschieden. Zwei Mischfuttermittel und die zugehörigen Einzelfuttermittel wurden ausgewählt, um die $InsP_6$-Konzentrationen und ED_{InsP6} zu charakterisieren. Analog zum XP- und ST-Abbau, wurde auch hier die Additivität und Wirkung des Pelletierens bewertet (Manuskript 3). Die Additivität war für ED_{InsP6} von Einzelfuttern in mehlförmigen Mischfuttermitteln, nicht aber in pelletierten Mischfuttermitteln gegeben. Das Pelletieren erhöhte signifikant die Werte für ED_{InsP6} von Mischfuttermitteln, obwohl der Effekt wahrscheinlich mit der Abnahme der Partikelgröße und dem höheren Verlust kleiner Partikel aus den *in situ*-Beuteln zusammenhängt.

In der vorliegenden Arbeit wurde die Schlussfolgerung gezogen, dass die Additivität des ruminalen Nährstoffabbaus und des Futterwerts von Einzelfuttermitteln in mehlförmigem und pelletiertem Mischfutter für die praktische Futterformulierung angenommen werden kann. Nur wenige assoziative Effekte, welche vermutlich auf methodische Ursachen zurückzuführen waren, konnten nachgewiesen werden.

ANNEX

Annex 1a. Comparison of ruminal crude protein effective degradability determined *in situ* (n = 3 cows) for passage rate of 8%/h ($ED_8CP_{IN\_SITU}$) calculated without and with lag phase

		ED_8CP no lag[1]	ED_8CP lag[2]	difference
Single feed				
Maize		62	61	-1
Wheat		82	81	-1
Barley		82	82	0
Soybeans		76	75	-1
Soybean meal		56	55	-2
Rapeseed meal		64	63	0
Sunflower meal		73	72	0
Faba beans		89	89	0
DDGS[3]		78	78	0
Maize gluten		87	87	0
Wheat bran		82	82	0
Sugar beet pulp		64	64	0
Compound feed				
1	mash	70	69	-1
	pellet	70	70	0
2	mash	76	76	0
	pellet	78	78	0
3	mash	84	84	0
	pellet	82	82	0
4	mash	72	72	0
	pellet	80	80	0
5	mash	67	67	-1
	pellet	73	72	0
6	mash	74	73	0
	pellet	77	77	0
7	mash	72	71	0
	pellet	74	74	0
8	mash	72	72	-1
	pellet	75	74	-1

[1]calculated according to Ørskov and McDonald (1979); [2]calculated according to McDonald (1981) modified by Südekum (2005); [3]DDGS, dried distillers' grains with solubles.

Annex 1b. Comparison of ruminal starch effective degradability determined *in situ* (n = 3 cows) for passage rate of 8%/h ($ED_8ST_{IN\_SITU}$) calculated without and with lag phase

		ED_8ST no lag[1]	ED_8ST lag[2]	difference
Single feed				
Maize		70	71	0
Wheat		97	97	0
Barley		92	92	0
Soybeans		-	-	-
Soybean meal		-	-	-
Rapeseed meal		-	-	-
Sunflower meal		-	-	-
Faba beans		90	93	-3
DDGS[3]		-	-	-
Maize gluten		86	86	0
Wheat bran		99	99	-1
Sugar beet pulp		-	-	-
Compound feed				
1	mash	81	81	0
	pellet	84	84	0
2	mash	86	86	0
	pellet	88	88	0
3	mash	92	92	0
	pellet	93	93	0
4	mash	88	88	0
	pellet	91	90	0
5	mash	78	80	-1
	pellet	82	82	-1
6	mash	84	87	-2
	pellet	87	87	0
7	mash	99	99	0
	pellet	97	98	-1
8	mash	94	95	-2
	pellet	95	96	-1

[1]calculated according to Ørskov and McDonald (1979); [2]calculated according to McDonald (1981) modified by Südekum (2005); [3]DDGS, dried distillers' grains with solubles.

Annex 2a. Overview of absolute and relative differences between calculated and observed protein values of compound feeds

	Absolute difference per compound feed								mean calc.	mean obs.	mean diff.	RMSE	RMSE % obs.
	1	2	3	4	5	6	7	8					
Ruminal CP degradation													
aCP_{IN_SITU} (%)	1	1	-2	-7	-2	-3	-4	-6	38	36	-3	3.0	8
bCP_{IN_SITU} (%)	0	-3	3	7	2	2	4	6	59	61	3	3.3	5
cCP_{IN_SITU} (%/h)	-9.6	-3.0	0.1	-9.4	-6.0	-3.9	-4.5	-4.9	20.1	15.0	-5.2	3.23	22
$lagCP_{IN\_SITU}$ (h)	-1.1	-0.8	-0.3	-1.2	-0.5	-0.5	-1.1	-0.9	1.2	0.4	-0.8	0.21	53
$ED_5CP_{IN\_SITU}$ (%)	0	2	1	-4	-3	-1	0	-1	80	80	-1	2.1	3
$ED_8CP_{IN\_SITU}$ (%)	1	3	1	-5	-3	-2	0	-1	74	73	-1	2.7	4
uCP_5 (g/kg DM)	-8	-13	-2	-6	-9	-7	-4	-4	192	186	-6	3.7	2
uCP_8 (g/kg DM)	-5	-10	-5	-6	-13	-12	-7	-4	216	208	-8	3.9	2
ID_{RUP} (%)	8	9	5	11	8	0	3	-4	53	58	5	5.5	10
CNCPS													
A (%)	4	7	1	-1	1	0	0	2	11	13	2	2.31	18
B1 (%)	-5	-8	-2	-1	0	2	0	0	16	15	-1	2.65	18
B2 (%)	2	1	2	3	-2	-1	-1	-2	62	62	0	1.92	3
B3 (%)	0	3	0	0	0	0	2	-1	7	8	0	0.87	11
C (%)	-1	-3	0	-1	1	-1	-1	0	3	3	-1	1.02	38
ED_{CNCPS}													
k = 5%/h (%)	2	5	11	8	1	6	5	6	74	80	6	3.4	4
k = 8%/h (%)	3	7	14	10	1	8	7	7	66	73	7	4.2	6

Observed (obs.) values for mash compound feeds are compared to values calculated (calc.) from single feeds. Positive values indicate observed values higher than calculated, negative values indicate observed values lower than calculated. RSME % obs. = root mean square error (RMSE) of the simple linear regression between calculated and observed values relative to the mean observed value across compound feeds. Ruminal crude protein (CP) degradation with degradation characteristics: a = rapidly degradable fraction; b = potentially degradable fraction; c = rate of degradation of b (%/h); lag = lag time; ED = effective degradation of CP at a passage rate of 5%/h (ED_5) and 8%/h (ED_8). uCP = utilisable crude protein for ruminal passage rates of 5%/h (uCP_5) and 8%/h (uCP_8); ID_{RUP} = intestinal digestibility of rumen undegraded protein; CNCPS = Cornell Net Carbohydrate and Protein System with fractions: A = non-protein nitrogen; B1 = rapidly degradable true protein; B2 = moderately degradable true protein; B3 = slowly degradable true protein, C = undegradable and indigestible true protein; and ED_{CNCPS} = Effective protein degradation for ruminal passage rates of 5%/h and 8%/h, calculated using Fox *et al.* (2003).

Annex 2b. Overview of absolute and relative differences between calculated and observed energy values and related values of compound feeds

	Absolute difference per compound feed											
	1	2	3	4	5	6	7	8	mean calc.	mean obs.	mean diff.	RMSE	RMSE % obs.
In vitro gas production													
*b*GP (ml/200 mg DM)	4	2	-1	2	1	1	4	3	67	69	2	1.7	2
*c*GP (%/h)	0.3	0.7	0.1	-0.7	0.5	-0.3	0.3	-0.3	8.1	8.2	0.1	0.44	5
GP_{24} (ml/200 mg DM)	1	0	-1	1	-1	1	0	2	62	63	0	1.2	2
dOM (%)	1	-1	-1	1	-2	0	0	2	86	86	0	1.3	1
ME[1] (MJ/kg DM)	-0.2	0.1	0.5	0.2	0.4	0.6	0.8	0.7	13.1	13.5	0.4	0.3	2
ME[2] (MJ/kg DM)	0.1	-0.1	-0.1	0.1	0.0	0.1	0.0	0.1	13.5	13.5	0.0	0.0	0
Ruminal ST degradation													
aST_{IN_SITU} (%)	-1	8	8	1	2	7	8	1	49	53	4	3.4	6
bST_{IN_SITU} (%)	1	-7	-7	-1	-2	-8	-9	0	50	46	-4	3.5	8
cST_{IN_SITU} (%/h)	-51.6	-120.6	-147.3	-19.0	-17.6	-11.0	3646	174	104.5	536.1	431.6	1065	199
$lagST_{IN\_SITU}$ (h)	0.0	0.0	-0.3	-0.2	0.5	0.6	-0.1	0.3	0.3	0.4	0.1	0.26	71
$ED_5ST_{IN\_SITU}$ (%)	1	1	1	0	0	0	1	1	90	91	1	0.5	1
$ED_8ST_{IN\_SITU}$ (%)	1	1	1	0	-1	0	2	1	87	87	1	0.7	1

Observed (obs.) values for mash compound feeds are compared to values calculated (calc.) from single feeds. Positive values indicate observed values higher than calculated, negative values indicate observed values lower than calculated. RSME % obs. = Root mean square error (RMSE) of the simple linear regression between calculated and observed values relative to the mean observed value across compound feeds. *b*GP = potential gas production; *c*GP = rate of gas production; GP_{24} = corrected gas production at 24 h; dOM = digestibility of organic matter; ME = metabolisable energy calculated from ME values of single feeds that were determined according to the equations of: 1) Krieg *et al.* (2017) and Menke and Steingass (1988), respective of the feed group; or 2) GfE (2009) for all single feeds; ruminal starch (ST) degradation with degradation characteristics: *a* = rapidly degradable fraction; *b* = potentially degradable fraction; *c* = rate of degradation of *b* (%/h); lag = lag time; ED = effective degradation of ST at a passage rate of 5%/h (ED_5) and 8%/h (ED_8).

Annex 2c. Overview of absolute and relative differences between calculated and observed ruminal dry matter degradation characteristics of compound feeds

	Absolute difference per compound feed								mean calc.	mean obs.	mean diff.	RMSE	RMSE % obs.
	1	2	3	4	5	6	7	8					
Ruminal DM degradation													
aDM_{IN_SITU} (%)	-3	0	0	-4	-3	-3	-3	-3	41	39	-2	1.5	4
bDM_{IN_SITU} (%)	3	-1	-1	5	3	3	2	4	50	52	2	1.9	4
cDM_{IN_SITU} (%/h)	-13.8	-13.7	-27.4	-30.7	-13.8	-18.9	-14.8	-24.9	19.9	0.1	-19.8	0.02	13
$lagDM_{IN\_SITU}$ (h)	4.6	-0.4	-0.2	-0.6	59.8	41.5	0.2	-0.9	0.6	13.6	13.0	23.68	174
$ED_5DM_{IN\_SITU}$ (%)	0	1	1	0	-2	-1	0	1	77	77	0	0.9	1
$ED_8DM_{IN\_SITU}$ (%)	0	2	1	-1	-2	-1	0	1	72	72	0	1.3	2

Observed (obs.) values for mash compound feeds are compared to values calculated (calc.) from single feeds. Positive values indicate observed values higher than calculated, negative values indicate observed values lower than calculated. RSME % obs. = root mean square error (RMSE) of the simple linear regression between calculated and observed values relative to the mean observed value across compound feeds. Ruminal dry matter (DM) degradation with degradation characteristics: *a* = rapidly degradable fraction; *b* = potentially degradable fraction; *c* = rate of degradation of *b* (%/h); lag = lag time; ED = effective degradation of DM at a passage rate of 5%/h (ED_5) and 8%/h (ED_8).

Annex 3. Contribution of *in vitro* determined GP_{24} values to the estimated values of digestibility of organic matter (dOM)

Compound feed	GP_{24} (ml/200 mg DM)	CP (g/kg DM)	CA (g/kg DM)	dOM[1] (%)	GP_{24} contribution to dOM (%)	GP_{24} contribution to dOM (%dOM)
1	73.9	16.1	3.1	93	74	79
2	65.5	17.7	4.9	86	65	76
3	61.1	19.7	5.0	83	61	74
4	66.9	21.9	3.6	90	67	75
5	61.7	23.6	4.5	86	62	72
6	58.8	25.2	5.3	84	59	70
7	54.3	27.8	5.9	81	54	67
8	59.1	30.1	5.0	87	59	68

[1]calculated using the equation of Menke and Steingass (1988): dOM (%) = 9.0 + 0.9991GP_{24} + 0.0595CP + 0.0181CA, where GP_{24} is corrected gas production at 24 h of incubation, CP is crude protein and CA is crude ash concentration; [2]absolute contribution of GP_{24} to dOM value; [3]relative contribution of GP_{24} to dOM value.

Annex 4. Comparison of calculated and observed ME values of compound feeds

Compound feed	ME[1] calculated	ME[2] calculated	ME[3] calculated	ME[4] observed
1	13.8	14.5	13.5	13.6
2	12.9	13.4	13.1	13.0
3	12.5	13.1	13.1	12.9
4	13.9	14.5	13.9	14.0
5	12.9	13.4	13.3	13.3
6	13.1	13.4	13.6	13.7
7	12.7	13.0	13.5	13.5
8	13.3	13.6	13.8	14.0

The ME values of compound feeds were calculated using different equations for ME of single feeds: ME[1], Krieg *et al.* (2017) or Menke and Steingass (1988) depending on the feed group; ME[2], Menke and Steingass (1988) for all single feeds; ME[3], GfE (2009) for all single feeds; ME[4], observed ME of compound feeds.

Annex 5a. Comparison of calculated and observed ruminal dry matter (DM) degradation characteristics of compound feeds in mash form determined *in situ* (n = 3 cows)

		DM					
Compound feed		*a*	*b*	*c*	lag	ED_5	ED_8
1	Calculated	45	52^{de}	13.9^{fgh}	0.5	79	73^{cde}
	Observed	42	55^{bc}	10.5^{h}	0.1	79	72^{de}
2	Calculated	44	48^{g}	13.8^{fgh}	0.4	77	71^{ef}
	Observed	44	47^{gh}	12.8^{gh}	0.0	78	73^{bcd}
3	Calculated	40	48^{gh}	27.7^{ab}	0.2	77	73^{bcd}
	Observed	40	47^{h}	22.4^{cd}	0.0	78	74^{bc}
4	Calculated	40	51^{def}	30.9^{a}	0.6	80	76^{a}
	Observed	36	56^{a}	18.6^{de}	0.0	80	75^{ab}
5	Calculated	39	52^{d}	13.9^{fgh}	0.7	74	68^{h}
	Observed	36	55^{ab}	10.8^{h}	0.6	72	66^{i}
6	Calculated	39	51^{f}	19.0^{de}	0.9	75	70^{g}
	Observed	36	54^{c}	13.6^{fgh}	0.4	74	69^{gh}
7	Calculated	45	45^{i}	15.0^{efg}	0.7	75	70^{gf}
	Observed	42	47^{h}	12.8^{gh}	0.0	75	70^{gf}
8	Calculated	39	51^{ef}	25.1^{bc}	0.9	77	72^{de}
	Observed	36	54^{bc}	17.2^{ef}	0.0	78	73^{cde}
Pooled SEM		-	0.6	1.99	-	-	0.9
CF	1	43				79^{b}	
	2	44				77^{c}	
	3	40				78^{c}	
	4	38				80^{a}	
	5	38				73^{e}	
	6	38				75^{d}	
	7	43				75^{d}	
	8	37				77^{c}	
Pooled SEM		-			-	0.6	
W	Calculated	41			0.6^{a}		
	Observed	39			0.1^{b}		
Pooled SEM		-			0.10	-	
p-	CF × W	-	<0.001	0.001	0.568	0.108	0.031
values	CF	-	<0.001	<0.001	0.100	<0.001	<0.001
	W	-	<0.001	<0.001	<0.001	0.862	0.499

a = rapidly degradable fraction (%); *b* = potentially degradable fraction (%); *c* = rate of degradation of *b* (%/h); lag = lag time (h); ED = effective degradation (%) of CP or ST at a passage rate of 5%/h (ED_5) and 8%/h (ED_8); pooled SEM, pooled standard error of the mean; different superscripts within a column and main effect (or their interaction) indicate significant differences; *p*-values, significance of main effects and their interactions: the way values were obtained (W: calculated and observed) and compound feeds (CF: 1–8).

Annex 5b. Effects of pelleting on ruminal dry matter (DM) degradation characteristics of compound feeds determined *in situ* (n = 3 cows)

		DM					
Compound feed		*a*	*b*	*c*	lag	ED_5	ED_8
1	Mash	42	55^{ab}	10.5	0.1	79^{cde}	72^{def}
	Pellet	45	51^{c}	13.1	0.0	81^{b}	76^{b}
2	Mash	44	47^{de}	12.8	0.0	78^{de}	73^{cde}
	Pellet	48	45^{i}	13.4	0.0	80^{bc}	75^{bc}
3	Mash	40	47^{efgh}	22.4	0.0	78^{de}	74^{bcd}
	Pellet	42	45^{hi}	20.7	0.0	79^{cde}	75^{bc}
4	Mash	36	56^{a}	18.6	0.0	80^{bc}	75^{bc}
	Pellet	44	48^{d}	21.8	0.0	83^{a}	79^{a}
5	Mash	36	55^{ab}	10.8	0.6	72^{j}	66^{i}
	Pellet	46	47^{defg}	9.6	0.2	76^{fgh}	71^{fg}
6	Mash	36	54^{b}	13.6	0.4	74^{i}	69^{h}
	Pellet	46	45^{ghi}	12.0	0.1	78^{efg}	73^{def}
7	Mash	42	47^{def}	12.8	0.0	75^{hi}	70^{gh}
	Pellet	48	42^{j}	11.0	0.1	76^{gh}	72^{efg}
8	Mash	36	54^{b}	17.2	0.0	78^{def}	73^{def}
	Pellet	46	46^{fghi}	14.1	0.0	79^{cd}	75^{bc}
Pooled SEM		-	0.6	-	-	0.8	1.0
CF	1	43		11.8^{cd}			
	2	46		13.1^{bc}			
	3	41		21.5^{a}			
	4	40		20.2^{a}			
	5	41		10.2^{d}			
	6	41		12.8^{cd}			
	7	45		11.9^{cd}			
	8	41		15.6^{b}			
Pooled SEM		-		1.7	-		
P	Mash	39					
	Pellet	45					
Pooled SEM		-		-	-		
p-	CF × P	-	<0.001	0.278	0.438	0.025	0.009
values	CF	-	<0.001	<0.001	0.062	<0.001	<0.001
	P	-	<0.001	0.611	0.178	<0.001	<0.001

a =rapidly degradable fraction (%); *b* = potentially degradable fraction (%); *c* = rate of degradation of *b* (%/h); lag = lag time (h); ED = effective degradation (%) of CP or ST at a passage rate of 5%/h (ED_5) and 8%/h (ED_8); pooled SEM, pooled standard error of the mean; different superscripts within a column and main effect (or their interaction) indicate significant differences; *p*-values, significance of main effects and their interactions: pelleting (W: mash and pelleted) and compound feeds (CF: 1–8).

Annex 5c. Comparison of calculated and observed dry matter disappearance (%) per incubation time point (h) in mash compound feeds determined *in situ* (n = 3 cows)

Compound feed		Incubation time point								
		0	2	4	6	8	16	24	48	72[1]
1	calculated	45	55	63	68	70	86	92	96	98
	observed	42	52	62	68	70	84	93	95	97
	difference	*-3*	*-3*	*-1*	*-1*	*0*	*-1*	*0*	*-1*	*-1*
2	calculated	44	55	62	67	71	84	88	92	94
	observed	44	57	64	68	76	85	88	91	92
	difference	*0*	*2*	*2*	*1*	*5*	*1*	*0*	*-1*	*-2*
3	calculated	40	61	68	72	76	80	85	90	97
	observed	40	61	67	74	78	81	84	89	91
	difference	*0*	*0*	*-1*	*2*	*2*	*0*	*0*	*-1*	*-6*
4	calculated	40	63	69	73	78	86	90	94	99
	observed	36	62	67	72	76	83	91	94	95
	difference	*-4*	*-1*	*-2*	*-1*	*-2*	*-2*	*2*	*1*	*-4*
5	calculated	39	48	56	63	67	81	88	91	96
	observed	36	45	53	62	65	77	88	90	92
	difference	*-3*	*-3*	*-4*	*0*	*-2*	*-4*	*0*	*-1*	*-4*
6	calculated	39	53	59	65	71	82	88	91	97
	observed	36	48	57	64	71	80	88	90	92
	difference	*-3*	*-4*	*-2*	*-1*	*1*	*-2*	*1*	*-1*	*-6*
7	calculated	45	54	59	65	71	82	86	89	95
	observed	42	53	60	68	70	83	85	88	89
	difference	*-3*	*-2*	*1*	*2*	*-1*	*1*	*-1*	*-1*	*-6*
8	calculated	39	56	62	68	74	83	88	91	100
	observed	36	55	62	72	75	86	87	91	92
	difference	*-3*	*-1*	*0*	*3*	*1*	*3*	*-2*	*-1*	*-7*

[1]Not all single feeds were incubated for 72 h. Disappearance of dry matter at 72 h was assumed to be 100% for those single feeds.

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 Anyukámnak,

Szeretném megköszönni a végtelen szereteted és türelmed anyu. Te voltál az aki megtanított arra hogyan legyek tisztességes és feleőségteljes, törekvő de nem törtető, úgy a munkámban, mint az életben. Folyamatosan támogattál, a nehéz időkben is, mikor a legnagyobb szükségem volt rá, ezt sosem felejtem el neked. Te tanítottál önállóságra, neked köszönhetem, hogy eljutottam idáig. Köszönöm hogy hittél bennem már a tanulmányaim végtelen ösvényének kezdetétől egészen máig. Csakúgy mint életemben, az első bizonyalan lépésektől, a cipőm befűzésén keresztül (sosem felejtem el), ezen disszertáció befejezéséig, mindig.

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Хвала ти, тата!

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_____

Goran Grubješić,
Stuttgart, 18th December 2019

DECLARATION IN LIEU OF AN OATH ON INDEPENDENT WORK

according to Sec. 18(3) sentence 5 of the University of Hohenheim's Doctoral Regulations for the Faculties of Agricultural Sciences, Natural Sciences, and Business, Economics and Social Sciences

1. The dissertation submitted on the topic:

"Investigations on ruminal degradation of nutrients and feeding values of single feeds and compound feeds for cattle."

is work done independently by me.

2. I only used the sources and aids listed and did not make use of any impermissible assistance from third parties. In particular, I marked all content taken word-for-word or paraphrased from other works.

3. I did not use the assistance of a commercial doctoral placement or advising agency.

4. I am aware of the importance of the declaration in lieu of oath and the criminal consequences of false or incomplete declarations in lieu of oath.

I confirm that the declaration above is correct. I declare in lieu of oath that I have declared only the truth to the best of my knowledge and have not omitted anything.

_____

Goran Grubješić,
Stuttgart, 18th December 2019